Effects of Imatinib on Monocyte-Derived Dendritic Cells Are Mediated by Inhibition of Nuclear Factor-κB and Akt Signaling Pathways

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ABSTRACT

Dendritic cells are the most powerful antigen-presenting cells playing a decisive role for the initiation and maintenance of primary immune responses. However, signaling pathways involved in the differentiation of these cells have not been fully determined. Imatinib is a novel tyrosine kinase inhibitor effective against Abl kinases, c-Kit, and platelet-derived growth factor receptor. Using this compound, we show that human monocyte-derived dendritic cells generated in the presence of therapeutic concentrations of imatinib show a reduced expression of CD1a, MHC class I and II, and costimulatory molecules as well as decreased secretion of chemokines and cytokines resulting in an impaired capacity of dendritic cells to elicit primary T-cell responses. Using Western blot analyses, we found that these effects are mediated by inhibition of phosphatidylinositol 3-kinase/Akt pathways and a pronounced downregulation of nuclear localized protein levels of nuclear factor-κB family members. Importantly, using blocking antibodies and tyrosine kinase inhibitors, we show that the inhibitory effects of imatinib on dendritic cell differentiation are not mediated via platelet-derived growth factor receptor and c-Kit. Taken together, our study reveals that imatinib inhibits dendritic cell differentiation and function via Akt and nuclear factor-κB signal transduction. Importantly, we show that imatinib can inhibit the function of normal, nonmalignant cells that may result in immunosuppression of these patients.

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INTRODUCTION

Dendritic cells are the most powerful antigen-presenting cells that play a crucial role for the initiation and maintenance of primary immune responses (1). Dendritic cells acquire antigens in the periphery and migrate to the lymph nodes where antigen specific T lymphocytes recognize the presented peptide antigens (1–3). Tissue-resident dendritic cells originate from bone marrow–derived circulating precursors able to develop into antigen-capturing dendritic cells (1, 2). *In vitro*, CD34^{+} progenitor cells can differentiate into dendritic cells after culture with different cytokine combinations, including granulocyte macrophage-colony-stimulating factor (GM-CSF), tumor necrosis factor-α, interleukin (IL)-4, Flt3 ligand, and stem cell factor. Alternatively, dendritic cells can be generated from CD14^{+} peripheral blood monocytes using GM-CSF and IL-4 (1, 2, 4–13). In a variety of immunotherapeutic approaches and in *in vitro* models, monocyte-derived dendritic cells (MDC) are used as antigen-presenting cells. Despite this broad application, the signal transduction pathways regulating the development and function of these cells have not yet been fully determined.

Imatinib (Glivec, Gleevec, STI571) is an ATP-competitive Bcr-Abl tyrosine kinase inhibitor that is also active against platelet-derived growth factor receptor (PDGF-R), c-Kit (14), Abl-related gene, and their fusion proteins while probably not affecting other tyrosine kinases (15–17). In clinical trials, imatinib has shown promising results in the treatment of Philadelphia chromosome–positive chronic myelogenous leukemia (CML; refs. 18–27), acute lymphoid leukemia (28, 29), and gastrointestinal stroma tumors (14, 15, 30–34) that are characterized by activating mutations of c-Kit as well as myeloproliferative disorders with mutations in the gene encoding PDGF-Rβ (35).

In this study, we show that exposure of human CD14^{+} peripheral blood monocytes to therapeutic concentrations of imatinib during differentiation into dendritic cells affects their phenotype, cytokine secretion, and T-cell stimulatory capacity due to inhibition of nuclear factor-κB (NF-κB) and Akt signaling pathways.

MATERIALS AND METHODS

Cells and Reagents. All cells were cultured in RP10 medium [RPMI 1640 with glutamax I, supplemented with 10% inactivated FCS and antibiotics (Invitrogen, Karlsruhe, Germany)]. Imatinib was a kind gift of Elisabeth Buchdunger (Novartis Pharmaceuticals, Basel, Switzerland). The cell lines used in the experiments were A498 (renal cell carcinoma, HER-2/*neu*[+], HLA-A2[+], DSMZ, Braunschweig, Germany), Croft (EBV-immortalized B cells, HER-2/*neu*[−], HLA-A2[+] kindly provided by O.J. Finn, Pittsburgh, PA), and SK-OV-3 (ovary

adenocarcinoma, HLA-A3$^+$, kindly provided by O.J. Finn). The CML cell line K562 was used to determine the natural killer cell activity.

Dendritic Cell Generation. Dendritic cells were generated from peripheral blood adhering monocytes by either magnetic cell sorting or plastic adherence as described previously (7, 36, 37). In brief, peripheral blood mononuclear cells were isolated by Ficoll/Paque (Biochrom, Berlin, Germany) density gradient centrifugation of blood obtained from buffy coats of healthy volunteers from the blood bank of the University of Tübingen. For magnetic cell sorting, CD14$^+$ magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used. For isolation of monocytes by plastic adherence, peripheral blood mononuclear cells were seeded (1×10^7 per well) into six-well plates (BD Falcon, Heidelberg, Germany) in X-VIVO 20 medium (BioWhittaker, Apen, Germany). After 2 hours of incubation at 37°C and 5% CO_2, nonadherent cells were removed and adherent monocytes were cultured in RP10 medium supplemented with GM-CSF (100 ng/mL, Leucomax, Novartis) and IL-4 (20 ng/mL, R&D Systems, Wiesbaden, Germany). Cytokines were added to differentiating dendritic cells every 2 to 3 days. Imatinib was dissolved in DMSO and added to the culture medium starting from the first day of culture together with GM-CSF and IL-4 in concentrations ranging from 1 to 5 μmol/L. To exclude effects induced by the solvent, equal amounts of DMSO were added as a control. In some experiments, dendritic cell maturation was induced by adding lipopolysaccharide (LPS, Sigma, Deisenhofen, Germany) at day 6 of culture. For blocking experiments, either antibodies or kinase inhibitors were added to the cells together with the cytokines. Antibodies used were directed against stem cell factor (mouse monoclonal, 1 μg/mL, R&D Systems), c-Kit (mouse monoclonal, 10 μg/mL, Sigma), PDGF, PDGF-Rα, and PDGF-Rβ (all goat polyclonal, 10 μg/mL, R&D Systems). Mouse IgG (Dianova, Hamburg, Germany) was added to control cells. Kinase inhibitors used were phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (100 nmol/L, Sigma), Src kinase inhibitor PP1, PDGF-R kinase inhibitor tyrphostin AG1296, and c-Abl kinase inhibitor AG957 adamantyl ester (all Calbiochem, Schwalbach, Germany). Because the substances were dissolved in DMSO, equal amounts of DMSO were added to control cells.

Immunostaining. Cells were stained using FITC- or phycoerythrin-conjugated mouse monoclonal antibodies against CD14, CD80, HLA-DR, CD3, CD11c, CD19, and CD54 (BD Biosciences, Heidelberg, Germany); CD33, CD40, and CD86 (BD PharMingen, Hamburg, Germany); CD1a, CD11a, and HLA-ABC (DAKO Diagnostika GmbH, Hamburg, Germany); CD83 (Immunotech, Marseilles, France); CCR7 (R&D Systems), and mouse IgG isotype control (BD Biosciences). Cells were analyzed on a FACSCalibur cytometer (BD Biosciences). A proportion of 1% false-positive events was accepted in the negative control samples. Staining was carried out in at least three independent experiments.

Detection of Apoptosis. For detection of apoptosis, the Annexin V-Fluos Staining kit (Roche Diagnostics GmbH, Mannheim, Germany) was used according to the instructions of the manufacturer in two independent experiments.

Cytokine Determination. Cytokine concentrations in supernatants from dendritic cell cultures were measured with commercially available two-site sandwich ELISA from R&D Systems (Rantes, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α) and Beckman Coulter (Hamburg, Germany; IL-12, tumor necrosis factor-α, IL-6) according to the manufacturer's instructions. Determination of cytokine and chemokine secretion was carried out in at least three independent experiments.

Induction of Antigen-Specific CTL Response Using the HLA-A2-Restricted Peptide E75 from HER-2/*neu*. The induction of HER-2/*neu*-specific CTL was done as described previously (36, 37). The HLA-A2 binding peptide derived from HER-2/*neu* E75 (369-377, KIGSFLAFL) and HIV (pol HIV-1 reverse transcriptase peptide, amino acids 476-484, ILKEPVHGV) were synthesized using standard Fmoc chemistry on a peptide synthesizer (432A, Applied Biosystems, Weiterstadt, Germany) and analyzed by reversed-phase high-performance liquid chromatography and mass spectrometry. For CTL induction, 5×10^5 dendritic cells were pulsed with 50 μg/mL E75 peptide for 2 hours, washed, and incubated with 3×10^6 autologous peripheral blood mononuclear cells without imatinib. After 7 days of culture, cells were restimulated with autologous peptide-pulsed peripheral blood mononuclear cells, and 2 ng/mL IL-2 (R&D Systems) was added on days 1, 3, and 5. Cytolytic activity of induced CTL was analyzed on day 5 after the last restimulation in a standard ^{51}Cr release assay.

CTL Assay. The standard ^{51}Cr release assay was done as described previously (7, 36, 37). Target cells were pulsed with 50 μg/mL peptide for 2 hours and labeled with ^{51}Cr sodium chromate (Amersham Biosciences Europe, Freiburg, Germany) for 1 hour at 37°C. Cells (1×10^4) were transferred to a well of a round-bottomed 96-well plate. Varying numbers of CTLs were added to a final volume of 200 μL and incubated for 4 hours at 37°C. At the end of the assay, supernatants (50 μL/well) were harvested and counted in a β-plate counter. The percentage of specific lysis was calculated as (experimental release − spontaneous release/maximal release − spontaneous release) × 100. Spontaneous and maximal releases were determined in the presence of either medium or 2% Triton X-100, respectively.

PAGE and Western Blotting. Nuclear extracts were prepared from dendritic cells as described previously (38, 39). For the preparation of whole cell lysates, cells were lysed in a buffer containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, and 1 mmol/L sodium orthovanadate. Protein concentrations of protein lysates were determined using a BCA assay (Pierce, Perbio Science, Bonn, Germany). For the detection of nuclear localized NF-κB family members, ~20 μg of nuclear extracts were separated on a 10% SDS-PAGE gel and transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Ponceau S staining of the membrane was done to confirm that equal amounts of protein were present in every lane. The blot was probed with antibodies against RelB (C-19, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), c-Rel (B-6, mouse

monoclonal, Santa Cruz), NF-κB p50 (C-19, goat polyclonal, Santa Cruz), or RelA (rabbit polyclonal, Upstate, Lake Placid, NY). For analysis of the activation state of mitogen-activated protein kinases p38 and extracellular signal-regulated kinase and Akt kinase, 20 to 30 μg whole cell lysates were separated on a 12% SDS-PAGE gel, transferred onto nitrocellulose membrane, and probed with phosphospecific antibodies phospho-p38 (Thr[180]/Tyr[182], rabbit polyclonal), phospho-p44/42 (Thr[202]/Tyr[204]), and phospho-Akt (Ser[473], mouse monoclonal, all Cell Signaling Technology-New England Biolabs, Frankfurt, Germany). As a control, antibodies against p38 mitogen-activated protein kinase (rabbit polyclonal, Cell Signaling Technology), extracellular signal-regulated kinase 1 (C-16, rabbit polyclonal, Santa Cruz), and Akt (rabbit polyclonal, Cell Signaling Technology) were used.

Incubation of all primary antibodies was followed by incubation with an appropriate horse radish peroxidase–conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence staining (Amersham Bio sciences Europe).

Western blotting was carried out in at least three independent experiments.

High-Density Oligonucleotide Microarray Analysis. Total RNA from dendritic cells generated in the presence or absence of 3 μmol/L imatinib was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer. Double-stranded cDNA was synthesized from 6 μg total RNA using SuperScript RTII (Invitrogen) and the primer (MWG Biotech, Ebersberg, Germany) according to the Affymetrix manual as described previously (40). *In vitro* transcription using the BioArray High-Yield RNA Transcript Labeling kit (ENZO Diagnostics, Inc., Farmingdale, NY), fragmentation, hybridization on Affymetrix U133A GeneChips (Affymetrix, Santa Clara, CA), and staining with streptavidin-phycoerythrin and biotinylated anti-streptavidin antibody (Molecular Probes, Leiden, the Netherlands) followed the manufacturer's protocols (Affymetrix). The Affymetrix GeneArray Scanner was used and data were analyzed with the Microarray Analysis Suite 5.0. For normalization, 100 housekeeping genes provided by Affymetrix were used (http://www.affymetrix.com/support/technical/mask_files.affx). Pairwise comparisons of dendritic cells generated in the presence versus absence of imatinib were calculated. Significance of differential expression was judged by the "change" values given by the statistical algorithms implemented in the Microarray Analysis Suite 5.0. For a gene to be considered as up-regulated or down-regulated, the following criteria had to be met: "increase" or "decrease" in the "change" value and presence in the "detection algorithm" in the sample with the higher expression. The complete experiment was done in duplicates.

Analysis of Phagocytic and Endocytic Capacity. For the analysis of phagocytic and endocytic activity, 2×10^5 cells were incubated with 3.4×10^7 FITC-labeled carboxylate-modified latex beads (1 μm, Sigma) or FITC-dextran (40,000 molecular weight, Molecular Probes-Invitrogen) for 1 hour at 37°C. As a control, 2×10^5 cells were precooled to 4°C before the incubation with latex beads or dextran at 4°C for 1 hour.

The cells were washed four times and immediately analyzed on a FACSCalibur cytometer. The results obtained represent three independent experiments.

RESULTS

Imatinib Inhibits the Differentiation of Peripheral Blood Monocytes into Dendritic Cells. Expression analysis of MDCs by reverse transcription-PCR revealed that all forms of PDGF as well as PDGF-Rβ but not PDGF-Rα are expressed in MDCs (data not shown). To determine whether PDGF and its receptor are involved in dendritic cell development from CD14^{+} monocytes, we analyzed if the presence of imatinib, a selective inhibitor of Bcr-Abl, c-Kit, and PDGF-R, would affect the differentiation of peripheral blood monocytes into dendritic cells.
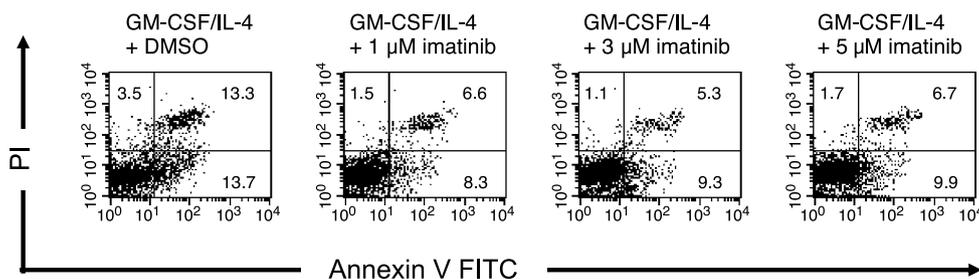
In the first set of experiments, we cultured CD14^{+} peripheral blood monocytes from healthy donors for 7 days with GM-CSF and IL-4. The cells differentiated into large, round, loosely adherent cells showing the typical cell protrusions in the form of dendrites. Phenotypic analysis of these cells by fluorescence-activated cell sorting staining showed acquisition of a dendritic cells phenotype characterized by CD1a and HLA-DR expression and loss of CD14 expression. Addition of imatinib in therapeutic concentrations varying from 1 to 5 μmol/L together with GM-CSF and IL-4 from the first day of culture did not affect the morphologic development but altered the dendritic cells phenotype. The cells were CD14 negative and characterized by reduced levels of CD1a and up-regulation of CD86 expression in a concentration-dependent manner (Fig. 1). This effect was also observed when imatinib was added to the medium of GM-CSF/IL-4 generated MDCs on day 4 of culture (Fig. 1). The expression of CD11a, CD11c, and CD33 was not affected (data not shown). Moreover, the phenotypic changes were not reversible, because additional culturing of the MDCs for 5 days without imatinib did not reestablish the expression of CD1a (data not shown). Importantly, the effects of imatinib were not caused by induction of apoptosis as analyzed by Annexin V and propidium iodide staining (Fig. 2).

Imatinib Inhibits Dendritic Cell Maturation. To evaluate the responsiveness of the MDCs generated in the presence of imatinib to a maturation stimulus, we added LPS 24 hours before harvesting the cells. In GM-CSF/IL-4-generated MDCs, this resulted in the up-regulation of the maturation marker CD83 as well as costimulatory molecules CD86, CD80, CD40, adhesion molecule CD54, and high expression levels of HLA-ABC and HLA-DR (Fig. 3). However, most of these molecules were not up-regulated in the presence of 3 μmol/L imatinib added from the first day of culture (Fig. 3). Similar effects on the expression of dendritic cell surface markers were observed when the medium was supplemented with imatinib after 4 days of culture (Fig. 3).

Imatinib Modulates Cytokine and Chemokine Secretion of Dendritic Cells. Secretion of cytokines and chemokines known to be important for dendritic cell function was analyzed by ELISA. Exposure of differentiating MDCs to imatinib inhibited monocyte chemoattractant protein-1 (CCL2) secretion while not affecting Rantes (CCL5), IL-6, or tumor necrosis factor-α production (Table 1). Dendritic cells differentiated in the presence of 3 μmol/L imatinib and stimulated with LPS

CD1a

CD86
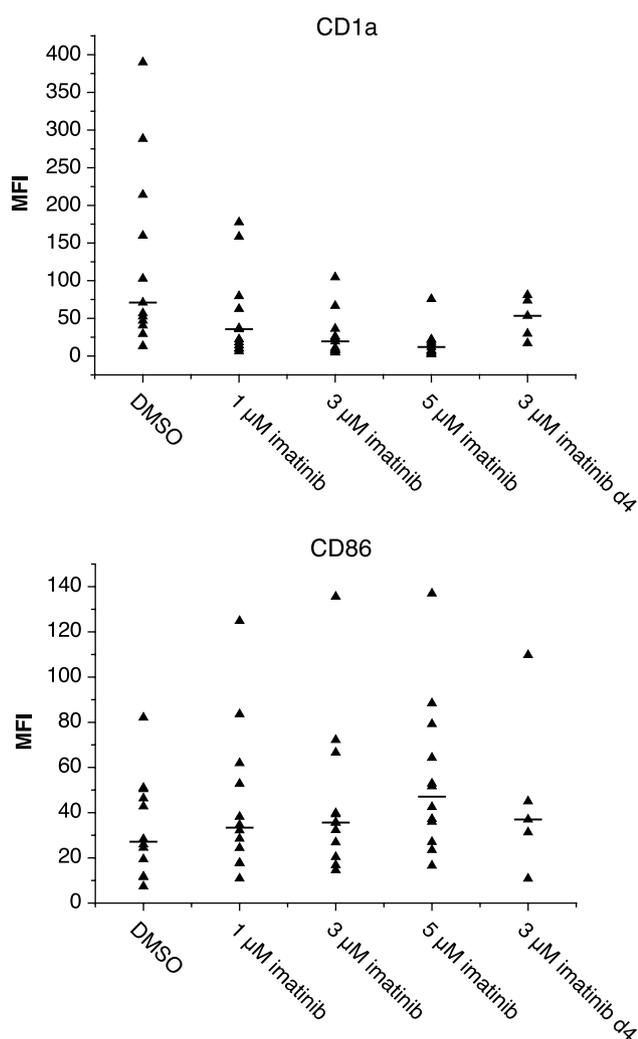
Fig. 1 Exposure of peripheral blood adhering monocytes to imatinib inhibits their differentiation into dendritic cells in a concentration-dependent manner. Peripheral blood adhering monocytes were cultured in the presence of GM-CSF and IL-4 for 7 days with or without imatinib in different concentrations (1−5 μmol/L) added from the first day of culture or on day 4 (3 μmol/L imatinib, day 4). Cells were analyzed by flow cytometry for expression of the dendritic cell marker CD1a and CD86. Mean fluorescence intensity of 12 independent experiments (*n* = 5 for 3 μmol/L imatinib day 4); median is indicated.

showed a reduction of monocyte chemoattractant protein-1 (CCL2), Rantes (CCL5), macrophage inflammatory protein-1α (CCL3), and IL-12 secretion, whereas the production of tumor necrosis factor-α, IL-10, and IL-6 was not altered (Table 2; data not shown). These inhibitory effects were also observed when imatinib was added on day 4 to the culture medium (Table 2).

Exposure to Imatinib Reduces the Capacity of Dendritic Cells to Initiate Specific Lymphocyte Responses. The induction of primary immune responses is a unique feature of dendritic cells. Therefore, we analyzed the ability of dendritic cells generated from adherent peripheral blood monocytes in the presence of GM-CSF and IL-4 with or without imatinib to initiate primary CTL responses. To accomplish this, MDCs were pulsed with the synthetic HLA-A2 binding peptide E75 derived from HER-2/*neu* tumor associated antigen and used as antigen-presenting cells. After two weekly restimulations, the CTL lines showed peptide specific and HLA-A2 restricted killing of tumor cell lines pulsed with the cognate peptide (Fig. 4*A*). This was also true for tumor cell lines constitutively expressing the HER-2/*neu* antigen (data not shown). Dendritic cells generated in the presence of 3 μmol/L imatinib failed to elicit antigen specific CTL responses (Fig. 4*B*). To ensure that this effect was not solely due to a reduced IL-12 secretion by MDCs treated with imatinib (Table 2), IL-12 was added during CTL priming. However, IL-12 supplementation did not restore the capacity of MDCs generated in the presence of imatinib to induce a primary CTL response (Fig. 4*C* and *D*).

The inhibitory effect of imatinib on the stimulatory capacity of dendritic cells was not due to an increased IL-10 secretion as analyzed by a commercially available ELISA (data not shown).

A Multitude of Genes Are Regulated by Imatinib. To further analyze which molecules and pathways are affected by imatinib and are probably involved in the observed inhibitory effects, we did comparative expression profiling using DNA microarray technology. Table 3 shows a selection of genes upregulated in dendritic cells treated with imatinib. Interestingly, most of the genes overexpressed after treatment of the cells with imatinib represent lysosomal proteins or molecules known to be primarily expressed in monocytes/macrophages, confirming the results obtained from the phenotypic analyses and indicating that imatinib inhibits the differentiation of monocytes into dendritic cells.
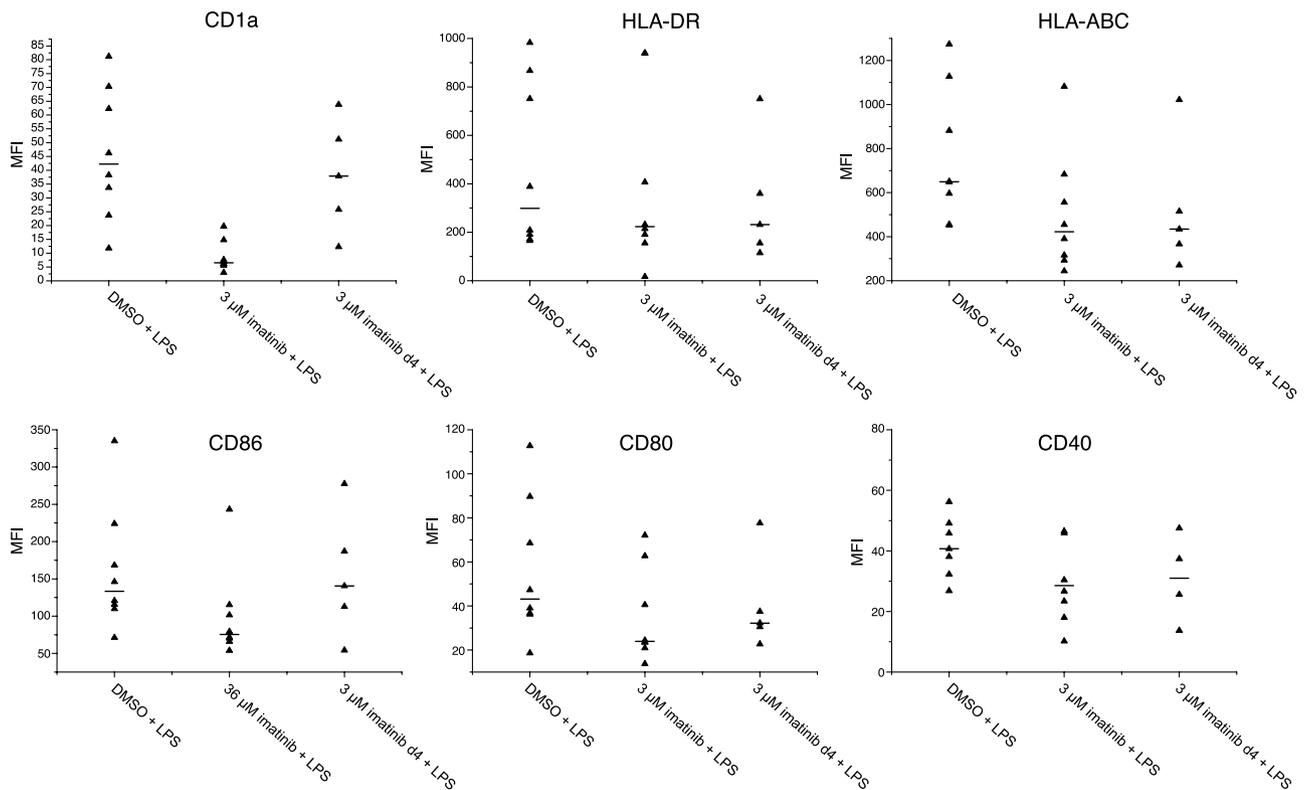

Fig. 2 Effects of imatinib are not mediated by induction of apoptosis. Peripheral blood adhering monocytes were cultured with GM-CSF and IL-4 in the presence or absence of varying imatinib concentrations (1−5 μmol/L). Viability of the cells and rate of apoptosis induction was analyzed by Annexin V-FITC and propidium iodide staining. Percentages of necrotic or apoptotic cells are indicated in the corresponding quadrants.

Fig. 3 Exposure to imatinib inhibits dendritic cell maturation. Peripheral blood adhering monocytes cultured with GM-CSF and IL-4 in the presence of 3 μmol/L imatinib added from the first day of culture or on day 4 were incubated with LPS as a maturation stimulus 24 hours before harvesting the cells. Effect of imatinib on dendritic cell phenotype was analyzed by fluorescence-activated cell sorting. Mean fluorescence intensity of 8 independent experiments (*n* = 5 for 3 μmol/L imatinib day 4 + LPS); median is indicated.

Imatinib Reduces Dextran Uptake by Dendritic Cells. Based on the results derived from gene expression and phenotype analysis, we sought to determine the phagocytic and endocytic capacity of the generated cell populations. To test this, we incubated the cells with FITC-labeled latex beads or FITC-labeled dextran for 1 hour at 37°C and 4°C. None of the cell populations were able to incorporate significant amounts of latex beads (data not shown). As shown in Fig. 5*A*, FITC-dextran accumulated in immature dendritic cells incubated at 37°C, whereas the control cells incubated at 4°C did not take up the dextran. Treatment of immature dendritic cells with 3 μmol/L imatinib resulted in a reduction of FITC-dextran uptake. As expected, mature dendritic cells stimulated with LPS only scarcely internalized FITC-dextran (Fig. 5*B*).

Imatinib Down-Regulates Nuclear Factor-κB Transcription Factor Family Members. Recently, it was shown that different members of the NF-κB family of transcription factors are important for the differentiation and function of dendritic cells (41–46). Therefore, we evaluated the nuclear localization of RelA, RelB, c-Rel, and NF-κB p50 proteins in the generated dendritic cell populations. Western blot analysis revealed that the amount of nuclear localized RelB (Fig. 6*A*), RelA (Fig. 6*B*), c-Rel (Fig. 6*C*), and NF-κB p50 (Fig. 6*D*) and the induction of these molecules by maturation stimuli is reduced in dendritic cells generated in the presence of 3 μmol/L imatinib added either from the first day of culture or

on day 4. These results suggest that the effects of imatinib are mediated at least in part by inhibition of NF-κB signaling pathways.

Imatinib Affects Phosphatidylinositol 3-Kinase Pathway. Regulation of NF-κB expression and function involves several pathways, including the mitogen-activated protein kinase and PI3K. To examine this, Western blot analyses and specific

Table 1 Impact of imatinib on cytokine and chemokine secretion of immature MDCs

	+DMSO	+Imatinib		
		1 μmol/L	3 μmol/L	5 μmol/L
Monocyte chemoattractant protein-1 (CCL2; pg/mL)				
Donor 1	>2,000	254.7	46.7	24.5
Donor 2	1,862.0	947.2	278.2	335.5
Donor 3	1,637.5	1,584.7	672.4	403.3
Rantes (CCL5; pg/mL)				
Donor 1	<0.1	<0.1	<0.1	<0.1
Donor 2	30.1	35.8	34.7	43.7
Donor 3	32.5	38.2	65.2	72.8
Tumor necrosis factor-α (pg/mL)				
Donor 1	7.1	5.3	6.1	3.4
Donor 2	19.2	14.7	13.4	9.9
Donor 3	58.6	43.9	49.4	59.3
IL-6 (pg/mL)				
Donor 1	<0.1	1.2	0.9	<0.1
Donor 2	12.6	12.9	16.1	17.1
Donor 3	5.5	6.3	5.2	8.1

Table 2 Impact of imatinib on cytokine and chemokine secretion of mature MDCs

	+DMSO	+Imatinib	
		3 μmol/L	3 μmol/L (day 4)
Monocyte chemoattractant protein-1 (CCL2; pg/mL)			
Donor 1	>10,000	4,172.5	4,820
Donor 2	>10,000	3,711.5	ND
Donor 3	>10,000	7,692.5	6,649.5
Rantes (CCL5; pg/mL)			
Donor 1	8,758	735	1,817
Donor 2	>20,000	7,865	ND
Donor 3	>20,000	6,837	9,687
Macrophage inflammatory protein-1α (CCL3; pg/mL)			
Donor 1	1,317	432	485
Donor 2	2,212	967	ND
Donor 3	4,430	1,850	1,652
IL-6 (pg/mL)			
Donor 1	3,121	3,023	2,975
Donor 2	3,107	2,954	ND
Donor 3	3,294	3,369	3,198
IL-12 (pg/mL)			
Donor 1	>1,000	43.9	425.6
Donor 2	>1,000	705.0	ND
Donor 3	801.9	18.2	91.3

kinase inhibitors were used to determine the possible involvement of these pathways in the inhibitory effects of imatinib. Using a phosphospecific antibody against Akt, concentration-dependent decrease of phosphorylation was observed (Fig. 7A). In contrast, we did not detect any differences in the phosphorylation state of p38 and extracellular signal-regulated kinase 1 (data not shown). To confirm that PI3K pathways are involved in the inhibitory effects imatinib has on the development and function of dendritic cells, PI3K inhibitor wortmannin was added to the cells starting from the first day of culture. The addition of this compound had a similar effect on dendritic cell phenotype as imatinib as analyzed by fluorescence-activated cell sorting staining (Fig. 7B).

Effect of Imatinib on Dendritic Cell Differentiation Is Not Mediated by c-Kit and Platelet-Derived Growth Factor Receptor. Besides Bcr-Abl, imatinib inhibits c-Kit and PDGF-R associated kinases. To analyze which tyrosine kinase is possibly involved in the observed inhibition of dendritic cell function, we did blocking experiments using neutralizing antibodies shown to be active as described previously (39, 47). Incubation of blood monocytes with antibodies against stem cell factor and its receptor c-Kit or antibodies against PDGF, PDGF-Rα, and PDGF-Rβ had no effect on dendritic cell development as analyzed by flow cytometry (data not shown), indicating that c-Abl inhibition is probably

Fig. 4 Induction of primary CTL responses by peptide-pulsed dendritic cells is impaired by addition of imatinib. Peripheral blood adhering monocytes were cultured with GM-CSF and IL-4 with or without imatinib (3 μmol/L). Dendritic cells were pulsed with the synthetic HLA-A2 binding peptide E75 derived from HER-2/*neu* tumor-associated antigen and used as antigen-presenting cells to induce a CTL response. Cytotoxic activity was determined after two restimulations in a standard ^{51}Cr release assay using Croft cells (HLA-A2[+], HER-2/*neu*[−]) pulsed with E75 (■) or HIV peptide (□) and K562 cells (▲) as target cells (*A* and *B*). Supplementation of IL-12 had no effect on the induction of primary CTL responses (*C* and *D*).
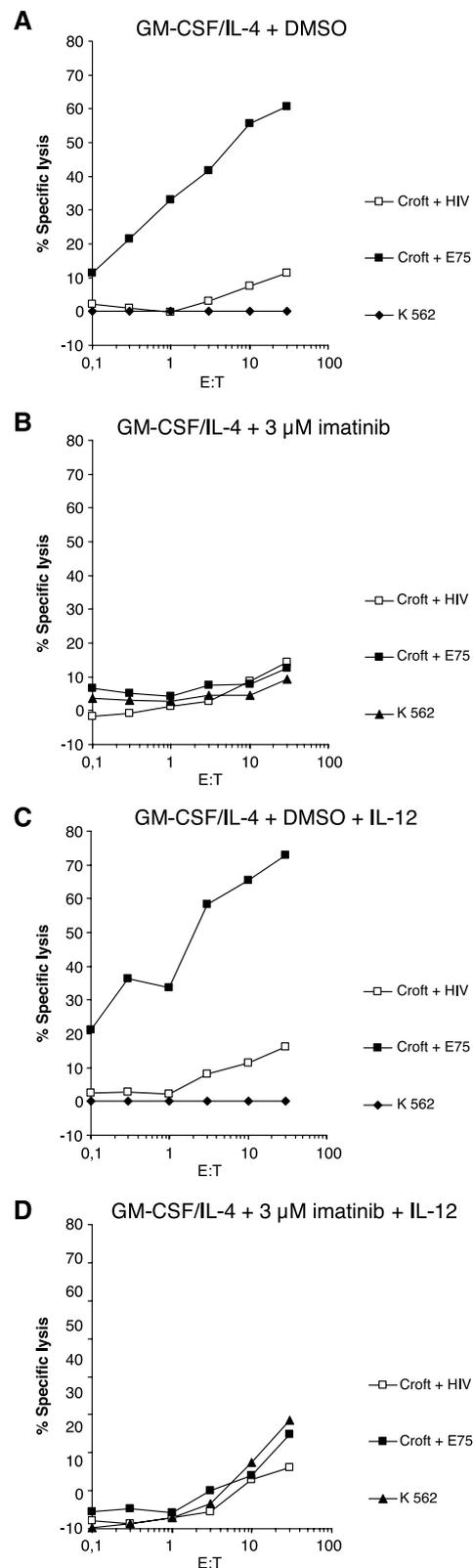
A — GM-CSF/IL-4 + DMSO

B — GM-CSF/IL-4 + 3 μM imatinib

C — GM-CSF/IL-4 + DMSO + IL-12

D — GM-CSF/IL-4 + 3 μM imatinib + IL-12

Table 3   Genes up-regulated by imatinib during dendritic cell differentiation

Gene*	Symbol	RefSeq[†]
Acid phosphatase 5, tartrate resistant	ACP5	NM_001611
Apolipoprotein C-I	APOC1	NM_001645
Apolipoprotein D	APOD	NM_001647
Apolipoprotein E	APOE	NM_000041
Cathepsin B	CTSB	NM_001908
Cathepsin D (lysosomal aspartyl protease)	CTSD	NM_001909
Cathepsin H	CTSH	NM_004390
Cathepsin L	CTSL	NM_001912
Cathepsin Z	CTSZ	NM_001336
CD68 antigen	CD68	NM_001251
CD88: complement component 5 receptor 1 (C5a ligand)	C5R1	NM_001736
CD105: endoglin (Osler-Rendu-Weber syndrome 1)	ENG	NM_000118
CD163: macrophage-associated antigen	CD163	NM_004244
CD204: macrophage scavenger receptor 1	MSR1	NM_002445
Fucosidase, α-L-1, tissue	FUCA1	NM_000147
Glucosamine (N-acetyl)-6-sulfatase (Sanfilippo disease IIID)	GNS	NM_002076
Glucosidase, β; acid (includes glucosylceramidase)	GBA	NM_000157
Glucuronidase, β	GUSB	NM_000181
GM2 ganglioside activator protein	GM2A	NM_000405
Hexosaminidase A (α polypeptide)	HEXA	NM_000520
Hexosaminidase B (β polypeptide)	HEXB	NM_000521
Leukocyte immunoglobulin-like receptor, subfamily B, member 3	LILRB3	NM_006864
Leukocyte membrane antigen	CMRF-35H	NM_007261
Lysosomal-associated membrane protein 1	LAMP1	NM_005561
Lysosomal-associated membrane protein 2	LAMP2	NM_002294
N-acylsphingosine amidohydrolase (acid ceramidase) 1	ASAH1	NM_004315
Oxysterol binding protein-like 3	OSBPL3	NM_015550
Peroxisome proliferative-activated receptor-γ	PPARG	NM_005037
Phospholipase D1, phophatidylcholine specific	PLD1	NM_002662
Phospholipid scramblase 1	PLSCR1	NM_021105
Protective protein for β-galactosidase (galactosialidosis)	PPGB	NM_000308
Retinoic acid receptor responder (tazarotene induced) 1	RARRES1	NM_002888
Sialidase 1 (lysosomal sialidase)	NEU1	NM_000434

*Gene symbols and names refer to LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/); whenever possible, symbols approved by the HUGO Gene Nomenclature Committee were used.

†RefSeqs are reference sequence standards provided by the National Center for Biotechnology Information.

involved in the observed alterations in dendritic cell phenotype and function. In the next set of experiments, we therefore used other tyrosine kinase inhibitors known to inhibit signaling mediated by the above-mentioned tyrosine kinases. As shown in Fig. 8*A* and *B*, addition of c-Abl inhibitor AG957 and Src kinase inhibitor PP1, which also inhibits c-Kit, PDGF-R, and

c-Abl (48), had similar effects on dendritic cell phenotype as shown for CD1a expression on dendritic cells as imatinib. Interestingly, incubation of peripheral blood monocytes with PDGF-R kinase inhibitor tyrphostin AG1296 did not significantly reduce the CD1a expression on dendritic cells, confirming that the effects of imatinib are not mediated by PDGF-R.

DISCUSSION

Although MDCs are extensively used as antigen-presenting cells in *in vitro* models and in a variety of immunotherapeutic approaches, signaling pathways mediating or affecting their differentiation have not been fully determined.

In our previous expression studies by reverse transcription-PCR, we found that PDGF-Rβ as well as its ligands are expressed in human MDCs (data not shown). Therefore, we used the specific
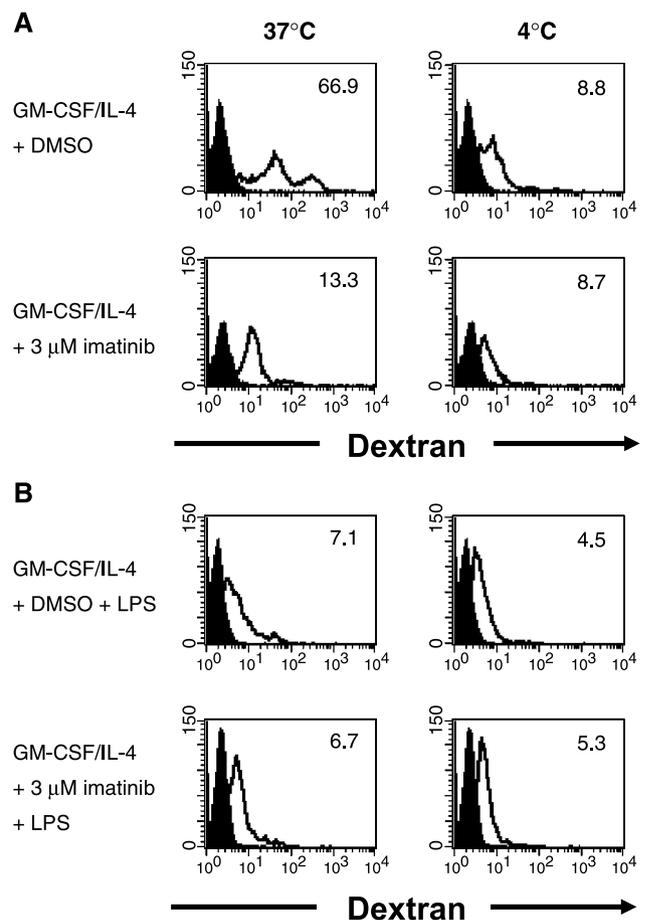
Fig. 5 Dextran uptake of dendritic cells is reduced by imatinib. Peripheral blood adhering monocytes were cultured with GM-CSF and IL-4 with or without imatinib (3 μmol/L) added from the first day of culture. For activation of dendritic cells, cells were incubated with LPS 24 hours before harvesting the cells. Dendritic cells (2×10^5) were incubated with FITC-dextran for 1 hour at 37°C, washed four times, and analyzed immediately on a FACSCalibur. As control, cells were precooled to 4°C and incubated with FITC-dextran for 1 hour at 4°C. Open histograms, FITC-dextran-treated cells; shaded histograms, untreated controls. Surface expression is mean fluorescence intensity: (*A*) immature dendritic cells and (*B*) mature dendritic cells.

A

B

C

D
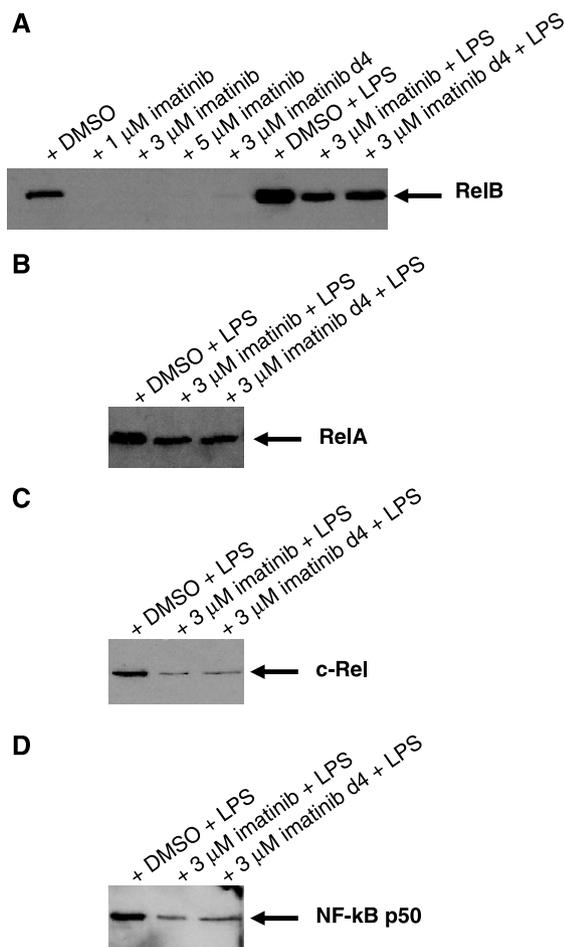
Fig. 6 Imatinib down-regulates the expression of nuclear localized RelB, RelA, c-Rel, and NF-κB p50 proteins in dendritic cells. Peripheral blood adhering monocytes were cultured with GM-CSF and IL-4 with or without imatinib (3 μmol/L) added from the first day of culture or on day 4. For activation of dendritic cells, cells were incubated with LPS 24 hours before preparing nuclear extracts. Nuclear localized RelB (*A*), RelA (*B*), c-Rel (*C*), and NF-κB p50 (*D*) proteins were detected by SDS-PAGE and Western blot. Ponceau S staining was carried out to confirm equal loading of the gel.

tyrosine kinase inhibitor imatinib to block this pathway during dendritic cell development *in vitro*. Imatinib is an ATP-competitive inhibitor of c-Kit, PDGF-R, Abl-related gene, and c-Abl as well as its fusion proteins, although it has no effects on other tyrosine kinases. Due to this selective inhibition, imatinib is currently used in the treatment of human malignancies caused by mutations resulting in an uncontrolled activation of these tyrosine kinases. Imatinib was shown to be very effective when applied to patients with CML, Philadelphia chromosome–positive acute lymphoid leukemia, or gastrointestinal stroma tumor by inducing a high rate of objective clinical responses with limited non-hematologic toxicity.

In our study, addition of imatinib to the medium in concentrations varying from 1 to 5 μmol/L, serum levels typically achieved in patients (18), resulted in the reduced expression of cell surface molecules known to be up-regulated or induced during the differentiation of monocytes into dendritic

cells (like MHC class I and II or costimulatory molecules) or represent dendritic cell–associated antigens like CD1a. The same effects were observed when imatinib was added after 4 days of culture, demonstrating that imatinib can also affect already differentiated dendritic cells. Interestingly, imatinib did not influence the viability of the cells as analyzed by Annexin V staining.

Further analysis of the functional capabilities of the cells generated in the presence of imatinib revealed that these cells showed an impaired ability to initiate primary T-cell responses and a reduced secretion of cytokines and chemokines induced on stimulation with LPS. This indicates that the inhibition of the functional properties of dendritic cells by imatinib is a result of down-regulated expression of MHC and costimulatory molecules and decreased activation-induced production of cytokines and chemokines involved in T-cell stimulation.

In the next set of experiments, we extended our analyses of the involved molecules and pathways by applying the DNA microarray technology. In these experiments, we found that treatment of cells with imatinib leads to up-regulation of lysosomal proteins and molecules primarily expressed in monocytes/macrophages. Therefore, we determined the phago-cytic and endocytic capacity of the generated cell populations. Although no effect on the phagocytosis rate as analyzed by latex beads uptake could be detected, immature dendritic cells incubated with 3 μmol/L imatinib had a reduced capacity to internalize FITC-dextran.

Interestingly, we also detected an increased expression of peroxisome proliferative-activated receptor-γ in imatinib-treated cells. Peroxisome proliferative-activated receptor-γ is a member of the nuclear hormone receptor superfamily, which are largely involved in the control of lipid metabolism and energy balance. Activation of peroxisome proliferative-activated receptor-γ by its ligands, the cyclopentenone prostaglandins of the J series, results in the inhibition of dendritic cell function (49, 50).

To further investigate the pathway of imatinib-mediated effects, we analyzed the nuclear localization of members of the NF-κB family known to be critically involved in dendritic cell differentiation and function. The Rel/NF-κB family of tran-scription factors regulates a broad variety of genes during immune responses (51) and has been shown to be essential for efficient antigen presentation by dendritic cells (41–46, 52–54). Activation of NF-κB is a complex process involving the phosphorylation of inhibiting IκB proteins by the IκB kinase complex resulting in the nuclear translocation of NF-κB. Mice with gene deletions of different NF-κB family members have helped to delineate their individual physiologic role (41, 55, 56). *In vitro* studies as well as RelB$^{-/-}$ mice showed that RelB is an important factor in dendritic cell function and development, as RelB$^{-/-}$ mice have no apparent myeloid dendritic cells (46). We described previously in line with the results from animal studies that RelB and c-Rel are involved or associated with the secretion of cytokines like IL-12 and the ability of dendritic cells to stimulate T cells (37). In the present study, we found by Western blot analyses a reduction of nuclear localized RelB in cells generated in the presence of imatinib and decreased activation induced up-regulation of RelB, RelA, c-Rel, and NF-κB p50, demonstrating that imatinib mediates its effects at least in part via the NF-κB pathway.
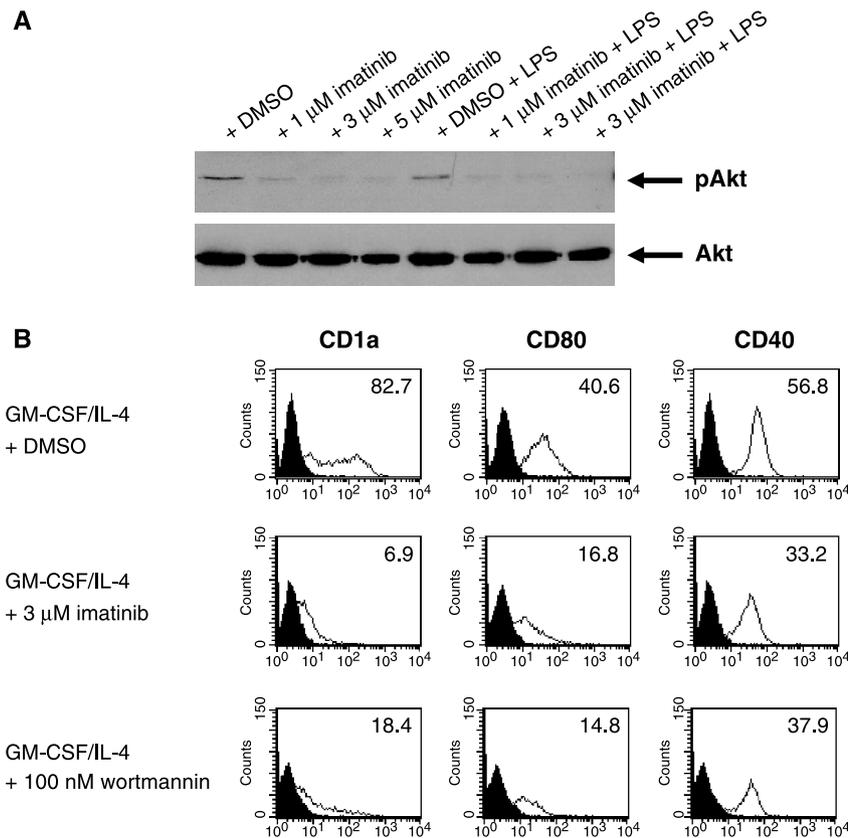
Fig. 7 Imatinib affects PI3K pathway. *A,* peripheral blood adhering monocytes were cultured with GM-CSF and IL-4 with or without imatinib in varying concentrations (1–5 μmol/L) from the first day of culture. For activation of dendritic cells, cells were incubated with LPS 24 hours before preparing whole cell lysates with phosphatase inhibitors. Phosphorylation state of Akt was detected by SDS-PAGE and Western blot using a phosphospecific antibody detecting phosphorylated Ser[473]. The blot was reprobed using an Akt antibody. *B,* peripheral blood adhering monocytes were cultured with GM-CSF and IL-4 with 3 μmol/L imatinib or 100 nmol/L PI3K inhibitor wortmannin. The phenotype of the generated cell populations was analyzed by fluorescence-activated cell sorting. Open histograms, staining with the indicated antibody; shaded histograms, isotype control. Surface expression is mean fluorescence intensity.

In line with these results, we observed that the presence of imatinib during dendritic cell generation reduced phosphorylation of Akt, which is a kinase involved in diverse pathways activating NF-κB (57–61). Moreover, PI3K inhibitor wortmannin had similar effects on dendritic cell phenotype as imatinib, confirming the involvement of this pathway.

In accordance with our results, it was shown recently by Dietz et al. that imatinib inhibits T-cell functions by affecting the NF-κB signaling pathway (62). Furthermore, the authors found that these inhibitory effects were accompanied by a diminished phosphorylation of extracellular signal-regulated kinase 1/2, which is in contrast to our results. However, higher concentrations of imatinib (10 μmol/L) were used in this study and analysis was done at earlier time points. In addition, these findings might reflect the use of different pathways in distinct cell populations.

In the next set of experiments, we tried to delineate which receptor tyrosine kinase might mediate the phenotypical and functional alterations induced by imatinib. Therefore, we first used blocking antibodies against c-Kit and its ligand stem cell factor as well as antibodies inhibiting all PDGFs and both receptors, PDGF-Rα and PDGF-Rβ. However, addition of these antibodies to the cell culture had almost no influence on dendritic cell phenotype, suggesting that the above-described effects of imatinib are probably mediated by inhibition of c-Abl or via other as yet unknown signaling cascades.
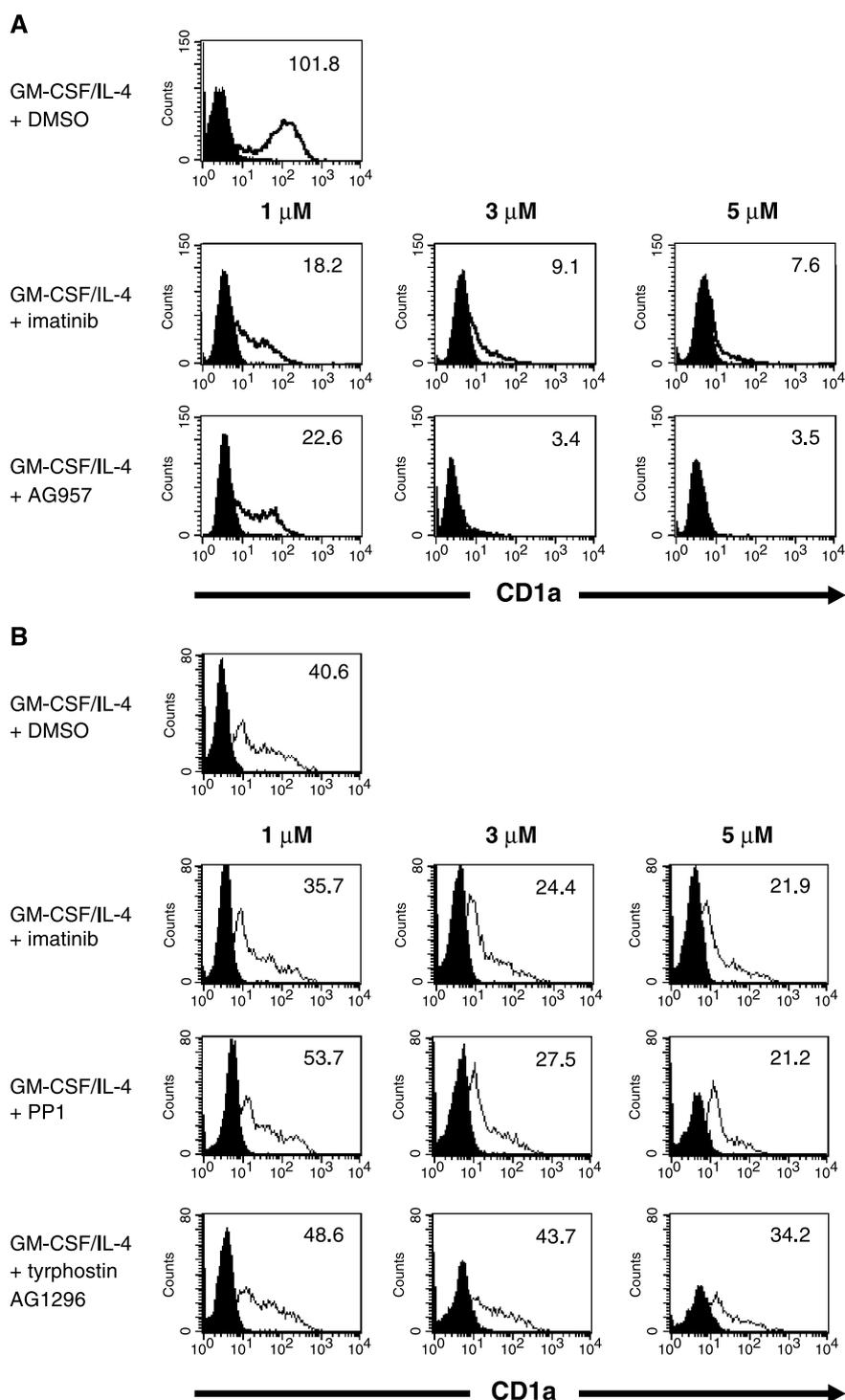
We next took advantage of other tyrosine kinase inhibitors: c-Abl-specific inhibitor AG957; PP1, a Src kinase inhibitor that also inhibits PDGF-R, c-Kit, and c-Abl tyrosine kinase (48); and tyrphostin AG1296, a selective inhibitor of PDGF-R. By applying these molecules, we found that AG957 and PP1, like imatinib, inhibits the up-regulation of CD1a in a concentration-dependent manner, whereas tyrphostin AG1296 had only marginal effect on dendritic cell phenotype, supporting the results from the experiments with the blocking antibodies.

We and others have shown previously that imatinib can act on normal CD34^{+} progenitor cells (47) and inhibit their development into dendritic cells (39). In contrast to monocytes and MDCs, CD34^{+} cells express c-Kit and stem cell factor, both of which have been shown to be important for the development of dendritic cells from human progenitor cells. However, in this study, we found that c-Kit is not significantly involved in the effects induced by imatinib. In contrast to the experiments with MDCs, we found that in CD34^{+}-derived dendritic cells imatinib only inhibited the up-regulation of RelB while not affecting RelA or c-Rel, indicating that the signal transduction pathways may be different in these cell populations.

In contrast to our findings, Sato *et al.* showed that antigen presentation of dendritic cells from CML patients is restored by addition of imatinib (63). However, Bcr-Abl-positive dendritic cells are functionally defective and differ from normal dendritic cells.

According to our results, c-Abl inhibition might be involved in the inhibitory effects of imatinib on dendritic cell function and differentiation. Although it is well analyzed that activated c-Abl has generally growth stimulatory and antiapoptotic effects, not much is known about the complex physiologic function of this nonreceptor tyrosine kinase in spite of multiple potential roles according to the functional domains of the

A

GM-CSF/IL-4
+ DMSO

GM-CSF/IL-4
+ imatinib

GM-CSF/IL-4
+ AG957

CD1a

B

GM-CSF/IL-4
+ DMSO

GM-CSF/IL-4
+ imatinib

GM-CSF/IL-4
+ PP1

GM-CSF/IL-4
+ tyrphostin
AG1296

CD1a

Fig. 8 Inhibitory effect of imatinib is not mediated by c-Kit and PDGF-R. *A,* peripheral blood adhering monocytes were cultured with GM-CSF and IL-4 with or without imatinib (1–5 μmol/L) and c-Abl kinase inhibitor AG957 (1–5 μmol/L), respectively, from the first day of culture. CD1a expression was determined by flow cytometry. Open histograms, staining with a CD1a antibody; shaded histograms, isotype control. Surface expression is mean fluorescence intensity. *B,* peripheral blood adhering monocytes were cultured with GM-CSF and IL-4 with or without imatinib (1–5 μmol/L), Src, c-Kit, PDGF-R and c-Abl kinase inhibitor PP1 (1–5 μmol/L), and PDGF-R inhibitor tyrphostin AG1296 (1–5 μmol/L), respectively, from the first day of culture. CD1a expression was determined by flow cytometry. Open histograms, staining with a CD1a antibody; shaded histograms, isotype control. Surface expression is mean fluorescence intensity.

protein. Studies in c-Abl knockout mice have shown that homozygous deletion of either the whole protein (64) or the COOH-terminal one-third (65) leads to neonatal lethality and B-cell and T-cell lymphopenias while not affecting myeloid or erythroid lineages. However, the development and function of dendritic cells in these animals were not analyzed. A more recent study has shown that c-Abl$^{-/-}$ mice are osteoporotic due to

defects in maturation of osteoblasts (66). In normal cells, involvement of c-Abl in cell division, cell differentiation, cell adherence, cell death, cell mobility, and integrin receptor-mediated signal transduction is likely (67–70).

In conclusion, we show that c-Abl tyrosine kinase might play an important role in the differentiation and function of MDCs and that imatinib mediates its effects at least in part via NF-κB and

PI3K/Akt kinase signal transduction pathways. Another important observation is that imatinib, a drug used in the treatment of patients with malignant diseases, can affect the function of normal, nonmalignant cells and might therefore result in immunosuppression in these patients. This is important as some of the therapeutic effects in the treatment of patients with CML are mediated by the induction of leukemia specific T-cell responses and imatinib is currently applied in the combination with allogeneic stem cell transplantation. This assumption is supported by the recent observation that the induction of myeloblastin-specific T cells is impaired in CML patients treated with imatinib in contrast to patients receiving IFN-α (71). Furthermore, mice treated with imatinib show a reduced Flt3 ligand-mediated expansion of dendritic cells and antitumor activity (72) as well as diminished delayed-type hypersensitivity responses *in vivo* (62). Moreover, several reports have been published that support the assumption of imatinib being immunosuppressive. Mattiuzzi *et al.* described that treatment of CML patients with imatinib is associated with an increased risk of herpes zoster infection (73). Other studies observed the induction of EBV-positive B-cell lymphoma (74), pulmonary alveolar proteinosis (75), and remission of rheumatoid arthritis (76) in CML patients during therapy with imatinib.

Taken together, our study shows that the inhibitory effects of imatinib on dendritic cell differentiation and function are mediated via Akt and NF-κB signaling pathways. Moreover, we show that imatinib can affect normal, nonmalignant cells, which may result in immunosuppression of patients treated with this drug.

ACKNOWLEDGEMENTS

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Effects of Imatinib on Monocyte-Derived Dendritic Cells Are Mediated by Inhibition of Nuclear Factor- κB and Akt Signaling Pathways

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