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A Nonredundant Role for Canonical NF-κB in Human Myeloid Dendritic Cell Development and Function

Lianne van de Laar,* Aniek van den Bosch,* Sandra W. van der Kooij,† Harry L. van Janssen,* Paul J. Coffer,‡,§ Cees van Kooten,† and Andrea M. Woltman*

The plastic role of dendritic cells (DCs) in the regulation of immune responses has made them interesting targets for immunotherapy, but also for pathogens or tumors to evade immunity. Functional alterations of DCs are often ascribed to manipulation of canonical NF-κB activity. However, though this pathway has been linked to murine myeloid DC biology, a detailed analysis of its importance in human myeloid DC differentiation, survival, maturation, and function is lacking. The myeloid DC subsets include interstitial DCs and Langerhans cells. In this study, we investigated the role of canonical NF-κB in human myeloid DCs generated from monocytes (monocyte-derived DCs [mo-DCs]) or CD34+ progenitors (CD34-derived myeloid DCs [CD34-mDCs]). Inhibition of NF-κB activation during and after mo-DC, CD34-interstitial DC, or CD34-Langerhans cell differentiation resulted in apoptosis induction associated with caspase 3 activation and loss of mitochondrial transmembrane potential. Besides regulating survival, canonical NF-κB activity was required for the acquisition of a DC phenotype. Despite phenotypic differences, however, Ag uptake, costimulatory molecule and CCR7 expression, as well as T cell stimulatory capacity of cells generated under NF-κB inhibition were comparable to control DCs, indicating that canonical NF-κB activity during differentiation is redundant for the development of functional APCs. However, both mo-DC and CD34-mDC functionality were reduced by NF-κB inhibition during activation. In conclusion, canonical NF-κB activity is essential for the development and function of mo-DCs as well as CD34-mDCs. Insight into the role of this pathway may help in understanding how pathogens and tumors escape immunity and aid in developing novel treatment strategies aiming to interfere with human immune responses. The Journal of Immunology, 2010, 185: 7252–7261.

Dendritic cells (DCs) are professional APCs that play a crucial role in the induction of immunity as well as tolerance (1). The DC subtype, its maturation state, and the life span of the Ag-bearing DC together determine the type of the initiated immune response (2–5). Based on surface markers, localization, functional abilities, and ontogeny, a large variety of DC subsets can be recognized (6).

The myeloid DC subtypes are known for their superior T cell priming ability compared with, for example, plasmacytoid DCs (7). Their ability to induce both immunogenic and tolerogenic immune responses has made them interesting tools for treatment of infections, cancer, autoimmune diseases, and allograft rejection (8). Much research has focused on the active manipulation of DCs, either to augment favorable immunity or to suppress unwanted immune activation (9–14). Also pathogens and tumors often escape immunity via modulation of DC immunogenicity (15–19). In many cases, the functional alterations of DCs by drugs, pathogens, or tumor-derived factors are ascribed to manipulation of NF-κB activity.

NF-κB/Rel transcription factor family members are expressed at relatively high levels in DCs (20). In mammals, they exist as homo- or heterodimers of five distinct proteins including RelA (p65), c-Rel, RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) (21). All of these proteins have been knocked out in mice, and deficiency of RelB or a combined deficiency of RelA and p50 or c-Rel and p50 all result in DC loss (22, 23). The noncanonical transcription factor RelB has further been shown to have a critical role in DC maturation and immunogenicity (22, 24–27). Canonical NF-κB activation, which involves phosphorylation and subsequent degradation of the inhibitory IκB proteins enabling nuclear translocation of RelA, c-Rel, and/or p50 dimers, has also been associated with activation-induced DC maturation and function (28–31).

Although knockout studies in mice have elegantly shown a loss of functional DCs in the absence of canonical NF-κB proteins, these experiments cannot be directly translated to the human situation. Furthermore, they do not allow separate analysis of the processes involved in DC biology. The exact mechanisms affected by the loss of NF-κB activity therefore remain undefined. Effects on survival, differentiation, maturation, and function can be investigated independently in vitro. In vivo, DCs develop from CD34+ hematopoietic progenitor cells (HPCs) via subset specific precursors that are present either as direct DC precursors, precursor DCs (pre-DCs), or as immune effector cells with their own function in immunity, such as monocytes (6, 32). To study human DC biology in vitro, DC differentiation from monocytes is the most widely used model (33). To investigate the development of
myeloid DC subsets from a less committed progenitor, CD34+ HPCs can be used. Two functionally different myeloid subsets, interstitial DCs (intDCs) and Langerhans cells (LCs), develop from CD34+ HPCs via their respective pre-DCs in independent pathways (34, 35), enabling detailed analysis of this DC developmental route.

Studies investigating canonical NF-κB activation in DC development have mainly focused on mouse DCs and human monocyte-derived DCs (mo-DCs). In the current study, we investigated the importance of canonical NF-κB activity in three different human myeloid DC subtypes: mo-DCs, CD34-derived intDCs, and CD34-derived LCs. A detailed analysis evaluating the role of canonical NF-κB in many different aspects of myeloid DC biology was performed. These data not only confirm previous studies suggesting that canonical NF-κB is involved in mo-DC function, but also show the importance of its activation for the functionality of other human myeloid DC subtypes. Moreover, we provide important information on the crucial role of canonical NF-κB in human myeloid DC differentiation and survival.

Materials and Methods

Reagents

Where indicated, the specific canonical NF-κB inhibitors caffeic acid phenethyl ester (CAPE; 10 μg/ml unless indicated differently; Sigma-Aldrich, St. Louis, MO), BAY 11-7082 (BAY; 1 μg/ml), curcumin (20 μg/ml; InvivoGen, San Diego, CA) to the cultures. Alternatively, cells were cocultured with CD40L-transfected L cells (L-CD40L) (40) in a DC/L cell ratio of 4:1. Nontransfected L cells (L-Orient) served as control cells. During activation, cells were incubated in the presence of 10 ng/ml GM-CSF and 10 ng/ml IL-4 (mo-DCs) or 100 ng/ml GM-CSF (CD34-derived myeloid dendritic cells [CD34-mDCs]). Cells and supernatants were analyzed after 48 h.

Allogeneic MLC

Responder T cells were isolated from a buffy coat. The mononuclear fraction was incubated with anti-CD15– and anti-CD235–coated microbeads (Miltenyi Biotec) and PE-labeled Abs against CD1c (AD5-B7E, Miltenyi Biotec), CD14, CD19 (J4.119, Beckman Coulter, Woerden, The Netherlands), CD56 (MY31, BD 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 (mo-DCs, 40 Gy; CD34-mDCs, 30 Gy) were added in graded doses to 15 × 10^6 (mo-DCs) or 2 × 10^6 (CD34-mDC) allogeneic T cells in 96-well round-bottom plates in RPMI 1640 containing 8% heat-inactivated FBS and penicillin/streptomycin. 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.

Cell phenotype analysis and apoptosis detection by flow cytometry

Surface markers. For phenotypic analysis, cells were washed in PBS containing 1% BSA, 1% heat-inactivated human serum, and 0.02% NaN3. Labeling of cell surface markers was performed on ice using fluorochrome-conjugated Abs against the following Abs: CD1a, CD14, CD19 (J4.119, Beckman Coulter), DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN; DC-SIGN; DCN46, BD Biosciences), CD40 (mAb89, Beckman Coulter), CD83 (HB15e, BD Biosciences), CD86 (Fun-1, BD Biosciences), B7-H1 (biotin-conjugated; M1H1, eBioscience), HLA-DR (L243, BD Biosciences), and CCR7 (150503, R&D Systems). Binding of biotin-conjugated Abs was visualized by a secondary incubation with fluorochrome-conjugated streptavidin (BD Biosciences).

Apoptosis. 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, and 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 μl/ml propidium iodide (PT, Sigma-Aldrich).

Mitochondrial transmembrane potential. Mitochondrial dysfunction was assessed using rhodamine 123 (Calbiochem). Cells were incubated in RPMI 1640 supplemented with 8% heat-inactivated FBS and penicillin/streptomycin at 37°C for 30 min in the presence of 0.1 μg/ml rhodamine 123. Then, cells were washed and taken up in PBS.

Caspase 3 activation. For intracellular staining of active caspase 3, cells were fixed (15 min) and subsequently permeabilized (5 min) by incubation in fixation medium and permeabilization medium (Fix&Perm, ADG Bio Research, Kaumorgen, Austria). Labeling of cleaved caspase 3 was performed on ice by 15 min incubation with FITC-conjugated rabbit anti-active caspase 3 (C92-605, BD Biosciences) in permeabilization medium. Rabbit serum was added to block aspecific labeling.

Assessment. Assessment was performed using a FACScalibur or FACS-Canto II (BD Biosciences), and data were analyzed using FlowJo software (http://www.flowjo.com; Tree Star, Ashland, OR).

Detection of cytokine production by ELISA

The commercially available ELISA kits for human IL-6, IL-10, IL-12p70, and IFN-γ (eBioscience) were used according to the manufacturer’s instructions. The detection limits of these assays were 2 pg/ml (IL-6 and IL-10) and 4 pg/ml (IL-12p70 and IFN-γ).

Western blot analysis

Cells were washed in PBS and lysed in NTEP-lysis buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, 5 mM EDTA, and 0.5% Nonidet P-40 supplemented with 1% Protease Inhibitor Cocktail (Sigma-Aldrich). Protein
concentrations were determined by the BCA protein assay (Pierce Chemical, Rockford, IL). Equal amounts of total lysate (20 μg/lane) were separated by 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) and incubated with blocking buffer (PBS, 0.1% Tween, 5% non fat dry milk, 1%BSA) before probing with Ab against IkBα (sc-371, Santa Cruz Biotechnology, Heidelberg, Germany). After subsequent incubation with HRP-conjugated Swine-anti-rabbit Ab (DakoCytomation, Glostrup, Denmark) and Supersignal (Pierce Chemical), blots were exposed to Hyperfilm films (Amersham Biosciences, Buckinghamshire, U.K.) to visualize labeled protein. Equal loading was confirmed by Coomassie blue staining.

Results

Canonical NF-κB activity regulates differentiation during mo-DC development

The role of canonical NF-κB in human myeloid DC development was first investigated in myeloid DCs generated from monocytes. To investigate the importance of canonical NF-κB transcriptional activity, its activation was inhibited by means of the pharmacological inhibitors CAPE, BAY, or AS. Effective blocking of NF-κB activity by these inhibitors was shown by abolished LPS-induced IkBα degradation and IL-6 production (Fig. 1). Whereas monocytes cultured with GM-CSF and IL-4 acquired a characteristic DC morphology and phenotype (CD14−CD1a+DC-SIGN+) within 6 d, inhibition of NF-κB by addition of 10 μg/ml CAPE, 1 μM BAY, or 1 μM AS during differentiation reduced the CD1a expression by day 6 monocyte-derived cells (Fig. 2A, 2B). This effect was dose-dependently reduced when the inhibitors were used at lower concentrations, whereas increased concentrations appeared extremely toxic (Fig. 2C). The induction of DC-SIGN expression was also affected by the inhibitors, whereas monocyte marker CD14 was downregulated in all conditions, and CD40 expression was normal regardless of the presence of NF-κB inhibitors (Fig. 2A, 2B). These data indicate that canonical NF-κB activity is required for complete mo-DC differentiation.

NF-κB inhibition allows differentiation of functional APCs

The functional abilities of the cells generated were investigated next. Cells differentiated in the presence of NF-κB inhibitors demonstrated normal endocytosis, as was apparent from the unaf-
fected uptake of Dextran\textsuperscript{FITC} (Fig. 3A). Although cells differentiated in the presence of CAPE showed a slightly higher CD86 and HLA-DR expression than control mo-DCs at the end of differentiation (day 6), this difference was lost after two additional days of culture in the absence of inhibitors, resulting in similar costimulatory molecule expression in all conditions (Fig. 3B and data not shown). LPS or CD40L stimulation in the absence of inhibitors induced upregulation of all costimulatory molecules, regardless of the presence of NF-κB inhibitors during differentiation (Fig. 3B). In addition, CCR7 expression could be induced in all conditions (Fig. 3B). When comparing LPS- and CD40L-induced cytokine production by cells differentiated in the presence of any of the three NF-κB inhibitors to cytokine secretion by control mo-DCs, no significant changes were observed due to high interdonor variability (Fig. 3C). Interestingly, however, when cells generated with different NF-κB inhibitors were analyzed together, and the inhibitor-induced alterations in CD1a expression, as typical readout for phenotypic differentiation, were combined with the induced modifications of IL-6, IL-10, or IL-12p70 production, a rather weak but significant correlation was found (Fig. 3C). Although these data suggest that, next to a clear effect on phenotype, differentiation of mo-DCs in the presence of NF-κB inhibitors might also modestly affect their LPS- and CD40L-induced cytokine production, the T cell stimulatory capacity of these cells was not significantly different from mo-DCs generated under control conditions. Both control and LPS-stimulated cells from all differentiation conditions induced similar T cell proliferation in allogeneic MLR (Fig. 3D). Although IFN-γ production by T cells cocultured with LPS-stimulated AS-differentiated cells was reduced compared with the control, overall, no changes in IFN-γ concentrations were observed (Fig. 3D). Thus, although phenotypic differences exist between mo-DCs differentiated in the presence or absence of NF-κB activity, NF-κB inhibition does not prevent the generation of functional APCs.

**FIGURE 3.** NF-κB inhibition allows differentiation of functional APC. CAPE, BAY, AS, or their solvent DMSO was added to mo-DC differentiation cultures at days 0, 2, and 5. A. Cells were harvested at day 6, thoroughly washed, and incubated with Dextran\textsuperscript{FITC} for 2 h at 37°C. Surface binding was determined by incubation at 4°C. Ag uptake was calculated as MFI 37°C – MFI 4°C. Cell debris, as determined by FSC/SSC, was excluded from the analysis. Data represent mean ± SEM Ag uptake standardized to control (n = 3). B. Day 6 cells were harvested, washed thoroughly, and counted with trypan blue exclusion. Equal cell numbers for all conditions were subsequently stimulated with or without 100 ng/ml LPS or L-CD40L. Cells were harvested after 48 h of stimulation and analyzed for the expression of CD86, HLA-DR, B7H1, and CCR7. Viability cells were gated on FSC/SSC. FACS plots representative of at least three experiments are shown. C. Day 6 cells were harvested, analyzed for CD1a expression by flow cytometry, washed thoroughly, and counted with trypan blue exclusion. Equal cell numbers for all conditions were subsequently stimulated with or without 100 ng/ml LPS or L-CD40L. Supernatants were harvested after 48 h of stimulation and IL-6, IL-10, and IL-12p70 concentrations were determined by ELISA. CD1a expression and cytokine concentration in supernatants were standardized to control. Data for all inhibitors were combined, and the Spearman’s rank correlation coefficient (r\textsubscript{s}) was calculated. Significance and r\textsubscript{s} are indicated. Data are derived from three to five experiments. D. Cells were harvested at day 6, washed thoroughly, and used in an allogeneic MLR immediately (n = 3) or after 24 h stimulation with LPS (n = 4). Equal DC/T cell ratios in all conditions were ensured by counting the cells with trypan blue exclusion just prior to coincubation. T cell proliferation was quantified by incubating the cells with [methyl-\textsuperscript{3H}]thymidine during the last 18 h of 6-d cultures. ELISA determined IFN-γ concentration in supernatants harvested at day 5. Proliferation and IFN-γ concentrations were standardized to control. Shown are mean ± SEM proliferation in cultures containing DC and T cells in a 1:30 ratio and mean ± SEM IFN-γ concentrations from cultures containing DC and T cells in a 1:10 ratio. *p < 0.05, paired Student t test.
Mitochondrial transmembrane potential was analyzed for mitochondrial transmembrane potential (ΔΨm) of CD1a+ cells showing active caspase 3 are shown (n = 4). Day 2 cells were analyzed for Annexin V/PI staining, and data were standardized to control cultures. Data shown represent mean ± SEM (n = 4). *p < 0.05, paired Student t test.

NF-κB inhibition results in apoptosis

Next to the effects on differentiation, NF-κB inhibition during mo-DC development also resulted in reduced cell yields at day 6 (Fig. 4A), indicating a potential role in the regulation of survival.

To investigate this further, mo-DC differentiation cultures were harvested at various time points and analyzed for apoptosis. Induction of apoptosis was clearly shown by increased Annexin V staining of the PI− population in inhibitor cultures compared with control at days 2, 3, and 6 (p < 0.05) (Fig. 4B and data not shown). In addition, activation of caspase 3 and reduced mitochondrial transmembrane potential was observed at all time points (Fig. 4C, 4D and data not shown). Together, these data show that NF-κB activation during mo-DC development is required to avoid the induction of apoptosis and ensure cell survival.

CD34-derived myeloid DC development requires canonical NF-κB

Next, we investigated the importance of NF-κB in the development of other myeloid DC types, the CD34-derived intDCs and LCs. Six days of culture in the presence of CAPE resulted in a large reduction in cell yield (98 ± 2%) (Fig. 5A). Within the viable cells, CAPE abrogated the generation of CD14−CD1a+ pre-intDCs from CD34+ HPCs, whereas the development of CD14−CD1a+ pre-LCs was relatively retained, as observed by flow cytometry 4 and 6 d after the start of culture (Fig. 5B, 5C). The combination of reduced yield and inhibited differentiation resulted in a strong dose-dependent reduction in pre-DC and pre-intDC numbers upon exposure to CAPE (Fig. 5D). These data show an important role for NF-κB in CD34-derived pre-DC development.

Next, differentiation of pre-DCs into terminally differentiated DCs was investigated. CAPE was added to FACS-sorted pre-intDCs or pre-LCs to separately examine the two individual subsets. Under control conditions, CD1a−CD14+ pre-intDCs differentiated to CD1a−CD14+−intDCs via an intermediate CD1a+CD14+ state, which was clearly present 48 h after pre-intDC isolation. In the presence of CAPE, however, CD1a+ cells did not appear (p < 0.01) (Fig. 6A). Forty-eight-hour cultures of pre-DCs resulted in >90% CD1a+ cells in the presence as well as in the absence of CAPE (Fig. 6A). These data suggest that NF-κB activity is required for intDC but not for LC differentiation. However, a significant reduction in viable cells was observed following CAPE treatment of both subsets (Fig. 6B). Already within 24 h, a 50 ± 9% and 54 ± 9% loss of viable cells was observed in intDC and LC cultures, respectively.
the next days, further cell loss became apparent, resulting in only 36 ± 12% and 18 ± 14% cells remaining after 3 d of treatment. Together, these data show that canonical NF-κB regulates differentiation as well as cell numbers during CD34-derived myeloid DC development.

**Induction of apoptosis during CD34-derived myeloid DC development**

As opposed to mo-DC cultures that lack proliferation, the observed cell loss in NF-κB inhibited CD34-derived myeloid DC cultures could result from inhibition of proliferation as well as induction of apoptosis. To investigate the role of apoptosis, NF-κB inhibitors CAPE or AS were added to CD34-myeloid DC cultures at various time points. As shown by the increased Annexin V binding (p < 0.05), apoptosis was induced upon NF-κB inhibition during both pre-DC development and terminal DC differentiation (Fig. 7A, 7B). Also, increased caspase 3 activation and loss of mitochondrial transmembrane potential was observed (Fig. 7C, 7D). Thus, the observed reduction of cells upon NF-κB inhibition can at least partly be explained by the induction of apoptosis.

**Myeloid DC survival and function requires canonical NF-κB**

Although we showed that NF-κB activity is required for survival and phenotypic differentiation of myeloid DCs, NF-κB inhibition during differentiation hardly affected the function of the APCs generated. To investigate whether NF-κB inhibition after terminal differentiation affects DC survival or function, CAPE or AS were added to fully differentiated mo-DCs, CD34-intDCs, and CD34-LCs. Apoptosis was induced upon 24- or 48-h culture in the presence of inhibitors (p < 0.05) (Fig. 8A), demonstrating differentiated myeloid DCs also require NF-κB activation to ensure their survival. Mo-DCs, CD34-LCs, and CD34-intDCs could be rescued from inhibitor-induced apoptosis by stimulation with CD40L, whereas LPS only prevented death of mo-DCs and CD34-LCs (data not shown). Both LPS and CD40L stimulation induced maximal expression of CD83, CD86, and HLA-DR in all cell types, independent of the presence or absence of NF-κB inhibitors (Fig. 8B and data not shown). B7H1, which was only expressed by mo-DCs, was also similarly expressed in all conditions. Whereas these data show that NF-κB is redundant for the induction of costimulatory molecule expression, NF-κB inhibition almost completely abrogated the induction of CCR7 expression by mo-DCs as well as CD34-mDCs (p < 0.05) (Fig. 8B), indicating that canonical NF-κB may be involved in the regulation of lymph node migration. Furthermore, inhibition of NF-κB activity significantly reduced 24 and 48 h activation-induced IL-6 and IL-12p70 secretion by all DC types (Fig. 8C and data not shown). In addition, T cell stimulatory capacity of myeloid DCs that were incubated with inhibitors prior to use in MLR was reduced compared with control DCs. Although no difference in T cell proliferation could be detected, the production of IFN-γ was reduced in MLR using DCs pretreated with CAPE or AS (Fig. 9). A similar pattern was observed at earlier time points (data not shown), indicating that the reduced IFN-γ concentrations resulted from reduced secretion rather than a delayed induction of T cell proliferation. Thus, canonical NF-κB activity regulates functional
maturation and thereby T cell stimulatory capacity of both mo-DCs and CD34-derived myeloid DCs.

Discussion
DCs are crucial in the induction of immunity as well as tolerance. Their development, survival, and maturation all critically influence the regulation of immune responses. Whereas mouse knockout studies have shown a role for the canonical NF-κB pathway in murine myeloid DC biology, a detailed analysis of its function in the different processes of functional human DC development is lacking. The present study investigated the role of canonical NF-κB activity in development and function of three different human myeloid DC subsets. By using several distinct pharmacological inhibitors the risk of observing non–NF-κB–related side effects was minimized. Overall, only minor differences between the inhibitors were apparent. Canonical NF-κB activity was found to regulate differentiation, survival, maturation, and function of mo-DCs as well as CD34-mDCs.

In mice, simultaneous deficiency of p50 and RelA has been shown to result in DC loss, but whether this was caused by inhibited differentiation or survival remained unclear (23). Canonical NF-κB plays a crucial role in antiapoptosis by the induction of antiapoptotic genes (41). Accordingly, its activity has been associated with augmented cell survival in various cell types (42–45). In line, in this study, we show that during development of human myeloid DCs, either from monocytes or from CD34+ HPCs, cell survival is strongly dependent on intact canonical NF-κB activation. Also survival of terminally differentiated mo-DCs and CD34-mDCs required NF-κB activity, though the cells could be rescued from inhibitor-induced apoptosis by the simultaneous presence of activation signals. Reduced survival due to decreased NF-κB activity has been described even in the presence of activation stimuli (23, 30, 46, 47), but these discrepancies probably result from differences in species, DC type, or experimental setup. Together, our data clearly show that canonical NF-κB plays a non-redundant role in the regulation of survival of differentiating as well as fully differentiated monocyte- and CD34-derived human myeloid DC.

Although apoptosis is traditionally thought to require caspase activation, caspase-independent apoptosis has also been described (48). In the current study, NF-κB inhibition-induced death was associated with phosphatidyl serine exposure, an archetypal caspase-dependent event (48, 49), and caspase 3 activation. The additional loss of mitochondrial transmembrane potential further implies a role for mitochondria in the induction and/or amplification of NF-κB inhibition-induced apoptosis. This is in accordance with the role of NF-κB in the induction of genes opposing caspase activation (50–52), as well as the transcriptional control of Bcl-2 family members (42, 53–55), for which an altered balance can lead to pore formation in the mitochondrial outer membrane, resulting in the release of apoptosis-promoting mitochondrial intermembrane space proteins (56).

As shown in this paper, not only survival but also differentiation of mo-DCs and CD34-derived intDCs depends on canonical NF-κB activity. Inhibited differentiation of CD34-derived intDCs upon NF-κB inhibition was demonstrated by the abrogated development of pre-intDCs and the inhibited CD1a acquisition during terminal differentiation. Similarly, the induction of CD1a and DC-SIGN expression on mo-DCs was reduced by specific NF-κB inhibitors, whereas loss of the monocyte marker CD14 occurred regardless of the presence of inhibitors. Reduced expression of differentiation markers was observed even when inhibitor-induced apoptosis was
FIGURE 9. NF-κB inhibition reduces T cell stimulatory capacity. Mo-DCs (A, B) were differentiated under control conditions until day 6 (n = 5). Control CD34-derived pre-intDCs and pre-LCs were isolated by FACS sort at day 6 and further cultured under control conditions until day 10 (LC) (C, D) or day 12 (intDC) (E, F) (n = 3). Terminally differentiated DCs were cultured with 100 ng/ml LPS in the presence of DMSO, CAPE, or AS. Cells were harvested after 24 h, were cultured with 100 ng/ml LPS in the presence of B, D, F). ELISA determined IFN-γ concentration in supernatants from the highest DC/T cell ratio, harvested at day 5. Concentrations were standardized to control. Mean ± SEM concentrations are shown (B, D, F). *p < 0.05, paired Student t test.

blocked by simultaneous addition of the pan-caspase inhibitor ZVAD-fmk (data not shown), indicating that the reduced differentiation was not merely a side effect of the presence of dying cells or debris. Furthermore, apoptosis induction in developing myeloid DCs is not necessarily associated with inhibited differentiation, as demonstrated in studies using inhibitors of unrelated pathways that affect survival but not differentiation of the developing DCs (39, 57). Thus, canonical NF-κB activation appears to be nonredundant in the differentiation of human myeloid DCs.

Despite their altered phenotype, cells differentiated under NF-κB inhibition showed normal Ag uptake and normal maturation upon TLR triggering or stimulation with T cell-derived signals such as CD40L. Functional analysis of CD34-derived myeloid DCs generated under NF-κB inhibition could not be performed due to massive cell loss, but for mo-DCs, Ag uptake capacity and induction of costimulatory molecules and CCR7 was similar for cells differentiated in the presence or absence of canonical NF-κB activity. When focusing on a single NF-κB inhibitor, cytokine production by mo-DCs generated in the presence of CAPE, BAY, or AS was not significantly different from control mo-DCs. Nevertheless, when combining all experiments and NF-κB inhibitors, a shift in cytokine profile partially related to inhibitor-induced phenotypic alterations could be observed. Although both the reduced expression of CD1a and DC-SIGN, molecules involved in Ag presentation and T cell stimulation (58, 59), and the altered cytokine production profile could influence the induction of T cell responses, no significant changes in allogeneic T cell stimulatory capacity were found. The unchanged T cell stimulatory capacity of mo-DCs generated with CAPE, BAY, or AS seems to be in contrast to the reported tolerogenic role of BAY (28). However, BAY only induced tolerance when it was used at a concentration of 2.5 μM or higher. At these concentrations, but not at the 1 μM concentration used in the current study, BAY also inhibited the noncanonical NF-κB protein RelB (28), a protein of crucial importance for DC immunogenicity. Thus, although noncanonical NF-κB is essential for the development of functional DCs, we show in this study that despite the role of canonical NF-κB proteins in the regulation of survival and phenotypic differentiation, the function of DCs differentiated in the presence of canonical NF-κB inhibitors was similar to control DCs.

As discussed above, canonical NF-κB inhibition during DC differentiation hardly affected the ability to respond to activation-inducing stimuli. The presence of canonical NF-κB inhibitors during activation of mo-DCs or CD34-mDCs, however, clearly inhibited DC function. In addition to previous studies on mouse myeloid DCs and human mo-DCs (30, 31, 60–63), the current study demonstrates that not only mo-DCs but also CD34-derived intDCs and LCs show reduced secretion of proinflammatory cytokines IL-6 and IL-12p70 as well as reduced T cell stimulatory capacity upon NF-κB inhibition during activation. The reduced activation-induced CCR7 expression in the presence of NF-κB inhibition could further abrogate DC functionality, as this could affect lymph node migration and hence interaction with T cells. As DC survival was unaffected in these cultures (data not shown), the reduced functionality reflects regulation of DC function by NF-κB, rather than side effects of cell death or reduced cell numbers. Ag uptake capacity was not affected by inhibition of NF-κB (data not shown). The unchanged upregulation of CD83,
Disclosures
The authors have no financial conflicts of interest.

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