Biochemical and Biophysical Research Communications **268,** 99–105 (2000)
doi:10.1006/bbrc.1999.2083, available online at http://www.idealibrary.com on IDEAL®

Differential Expression of the Transcription Factor NF-κB during Human Mononuclear Phagocyte Differentiation to Macrophages and Dendritic Cells[1]

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Received November 29, 1999

An important role for the Rel/NF-κB family of transcription factors in the differentiation process of dendritic cells (DC) and macrophages (MAC) was recently suggested by a number of mouse knockout studies but only little information is available for defined populations of human cells. To investigate the role of individual NF-κB proteins [p50, p52, p65 (RelA), RelB] in the differentiation of monocyte-derived cell types we analyzed and compared the expression pattern and DNA binding activity of NF-κB members in human monocytes (MO), MO-derived MAC, and MO-derived DC. Constitutive expression of p65 and RelB mRNA was found in MO and no significant regulation was observed during differentiation of MO into MAC or immature DC. Only during lipopolysaccharide-induced terminal differentiation of DC was a marked increase in RelB mRNA detected. In DNA binding assays performed with nuclear extracts from blood MO, p50/p50 homodimers were mainly detected, whereas complexes containing p50/RelB and p50/p65 heterodimers were less abundant. DNA-bound protein complexes containing p50/RelB and p50/p65 increased and additional p65/p65 complexes appeared during differentiation of MO into either MAC or immature DC. A strong increase in complexes containing p50/RelB was observed during terminal differentiation of DC. Therefore, gradual differences in the DNA binding activities of different NF-κB homo- and heterodimers correlate with differentiation stages of MO, MAC, and DC and are probably important for the biological role of these cells.

Key Words: **monocyte; macrophage; dendritic cell; NF-κB; RelB.**

Nuclear factor kappa B (NF-κB) is a potent transcription factor that is found in a great variety of immune cells and is known to participate in the regulation of multiple genes involved in development and immunity. NF-κB was originally identified as a protein binding to a specific decameric DNA sequence within the intronic enhancer of the immunglobulin kappa light chain gene in mature B- and plasma cells (1). To date five structurally related proteins of the Rel/NF-κB family have been identified in mammals: p50, p52, p65 (RelA), RelB and c-rel. They all contain a conserved domain of 300 aa in length, the Rel homology domain, which is responsible for dimerization and DNA binding (2). Various dimer combinations have been described and Rel/NF-κB proteins were classified into two groups. The first one comprises p65, RelB, and c-rel, which contain transactivation domains in their C-terminal sequences (3). In contrast, the members of the second group, p50 and p52, lack C-terminal transactivating regions and therefore are considered not to be transcriptionally active. Cell stimulation leads to a phosphorylation of the inhibitors of Rel/NF-κB [IκBs, reviewed in (4)] through specific IκB kinases (IKK) (5) and dissociation of IκB from the complex. The resulting free NF-κB dimers translocate into the nucleus and induce gene transcription [reviewed in (4)]. In addition, constitutive Rel/NF-κB DNA binding without specific cell stimulation has also been reported predominantly for heterodimers containing RelB (6).

Mononuclear phagocytes participate in both specific and nonspecific immune responses and play a critical role in extracellular matrix remodeling and wound healing (7). Peripheral blood monocytes (MO) provide the common precursor of tissue macrophages (MAC), the latter being considered mature effector cells. Under *in vitro* conditions, MO adhere tightly to cell culture substrates and a similar process of maturation from human blood MO to MAC takes place, accompanied by specific changes in morphology, antigenic expression and functional properties (8). Both MO and mature

[1] This work was supported by Deutsche Forschungsgemeinschaft (DFG AN 111/6-6).
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0006-291X/00 $35.00

MAC can be activated by proinflammatory stimuli, one of the most potent being bacterial endotoxin (lipopolysaccharide, LPS). In the last few years a close relationship between dendritic cells (DC) and the MO/MAC system was postulated and it was shown, that under appropriate conditions, DC can also differentiate from MO (9). Dendritic cells play a central role connecting nonspecific and specific immune responses by taking up antigen and inducing a specific T lymphocyte response. Evidence is coming up that the MO differentiation pathway to DC is also operative *in vivo* (10), indicating a dichotomy of MO differentiation either into antigen presenting cells leading to specific immunity or into strongly phagocytosing, classical MAC.

Several reports show an important role of NF-κB in human MO, MAC and DC. NF-κB is involved in the transcriptional regulation of immediate early genes in MO including cytokines like IL-6 (11) or TNF-α (12). Furthermore, production of the vast repertoire of cytokines by MAC is in part controlled by the DNA binding of NF-κB proteins (13) and several MAC associated molecules contain putative NF-κB binding sites in their promoters (14) indicating a wide immunemodulatory capacity of NF-κB in human MAC. In addition, different genetic approaches in mice [reviewed in (15)] reveal strong evidence for an essential role of NF-κB family members in the development and function of MO and MAC. For example, MAC of p50 knockout mice are impaired in elimination of intracellular bacteria (16). p50/p52 double knockout mice develop osteopetrosis due to a lack of osteoclasts (a specialized type of MAC) and their tissue MAC are functionally defective (17), indicating the importance of these molecules in MAC differentiation. Data from RelB knockout mice (18) which showed that RelB is required for the development of thymic medulla and of DC, implicate a key role especially of this member of the NF-κB family for DC differentiation. In contrast to these data from knockout mice, only little information on the distribution of individual Rel/NF-κB molecules in human MO, MAC and DC is available. Therefore we investigated the expression and DNA binding activity of Rel/NF-κB family members during the differentiation of MO into either mature MAC or DC.

MATERIALS AND METHODS

Cell isolation and culture. Peripheral blood mononuclear cells (MNC) were isolated from leukapheresis concentrates of healthy donors by density gradient centrifugation over Ficoll/Hypaque. MO were separated from MNC by counter-current elutriation in a J6M-E Beckman centrifuge with a large chamber and a JE-5 rotor at 2500 rpm and a flow rate of 110 ml/min with Hank's balanced salt solution as described previously (19). Elutriated MO were more than 90% pure as determined by morphology and antigen expression measured by flow cytometry.

For *in vitro* differentiation of MO to MAC purified MO were cultured on hydrophobic Teflon foils for 7–8 days at a cell density of 10^6 cells/ml in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with mercaptoethanol, polyvitamins, antibiotics, pyruvate, nonessential amino acids (all from Gibco BRL, Eggenstein, Germany) and with 2% human pooled AB-group serum as described previously (20). For generation of MO-derived DC, MO were cultured for 7–8 days in tissue culture plates in supplemented RPMI 1640, containing 500 U/ml interleukin-4 (IL-4, Promocell, Heidelberg, Germany), 40 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF, Essex, Munich, Germany) and 10% fetal calf serum (FCS, Gibco BRL). All reagents used were considered to be free of LPS contamination by measuring no spontaneous IL-6 secretion of freshly isolated MO. In some experiments LPS, 50 ng/ml, (from Salmonella abortus equi, kind gift from C. Galanos, Freiburg) was added to the cultures as indicated in the figures.

RNA preparation and Northern blot analysis. Total RNA was isolated at different time points from freshly isolated MO, adherent MO, *in vitro* differentiated MAC or *in vitro* differentiated DC by the guanidine thiocyanate/acid phenol method (21). For Northern blot analysis RNA samples (10 μg/lane) were separated by electrophoresis on 1% agarose/formaldehyde gels, transferred to nylon membranes (Magna NT, MSI, Westbrough, MA) and UV cross linked. Hybridization and washing were performed as described (22) using ^{32}P-labeled cDNA fragments (Random Primed DNA Labeling Kit, Boehringer, Mannheim, Germany). To provide an internal control, membranes were reprobed with an oligonucleotide against 18S rRNA labeled by T4-Kinase (5′-end labeling kit, Amersham, UK). Autoradiography was performed at −70°C.

Signal intensities of the RNA bands were measured by a personal densitometer (Molecular Dynamics, Sunnyvale, CA) and values divided by the signal intensities measured on 18S rRNA autoradiographs in order to correct for the RNA quantities loaded.

Preparation of nuclear extracts. Nuclear and cytosolic extracts for electrophoretic mobility shift assays (EMSAs) were generated using a slight modification of the protocol described by Schütze and co-workers (23). Cells (5×10^6 to 10×10^6) were harvested after the various time periods, washed twice in ice-cold PBS and suspended in ice-cold cytoplasmic extraction buffer (CEB; 10 mM Tris–HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol). After equilibration in CEB for 5 min, the cells were collected by centrifugation and lysed in 50 times the packed cell volume of NP-40/CEB/PI (CEB containing 0.4% Nonidet-40 [NP-40] and protease inhibitors 1 mM phenylmethylsulfonyl fluoride [PMSF], 50 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 40 μg/ml bestatin, 3 μg/ml E64, 1 mM 1,10-phenanthrolene, and 100 μg/ml of chymostatin; all from Boehringer, Mannheim, Germany). After 3 min nuclei were pelleted and the supernatant (cytoplasmic extract) collected and frozen. Isolated nuclei were washed once with cold CEB containing protease inhibitors but no detergent, suspended, and then mixed with 50 μl of nuclear extraction buffer (NEB; 20 mM Tris–HCl pH 8.0, 0.4 M NaCl, 1.5 mM $MgCl_2$, 1.5 mM EDTA, 1 mM dithiothreitol, protease inhibitors as in CEB, and 25% glycerol). After 10 min incubation on ice the samples were centrifuged and the supernatant (nuclear extract) snap frozen in liquid nitrogen before storage at −70°C. Protein concentrations were determined by the bicinchoninic acid method (Pierce).

Electrophoretic mobility shift assay (EMSA). EMSAs were performed as previously described (24). Briefly, DNA-protein binding reactions were performed in 10 mM Tris–HCl pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5 μg of bovine serum albumin (BSA), 0.6 μg of poly (dI–dC), and 4% Ficoll in a final volume of 25 μl. Each reaction contained 0.5 μg protein of nuclear extract and 0.3 ng labeled DNA corresponding to the NF-κB binding site of the major histocompatibility complex enhancer (5′-GAATTCGGCTGGGGATTCCCCATCTA-3′). In parallel, specific NF-κB subunits were identified in shifted complexes by using specific supershifting antibodies (all from Santa Cruz) against p50, p52, p65 (RelA), and RelB components of NF-κB. Densitometric evaluation of the individual bands

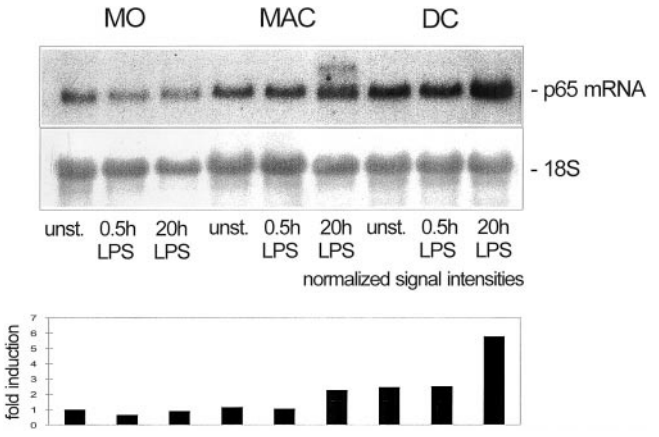

FIG. 1. Expression of p65 mRNA in unstimulated and LPS-stimulated MO, MAC, and DC. MO, MAC, or DC were harvested unstimulated or after stimulation with LPS for 0.5 or 20 h. Total RNA was prepared, 10 μg/lane were loaded and analyzed for p65 mRNA expression by Northern blot analysis. As an internal control the membrane was reprobed with an 18S rRNA oligonucleotide. Normalized signal intensities are shown below the blots. One representative experiment out of two is shown.

was performed using films with short exposure times to guarantee nonlinear signals. The width of the bands was taken into account for the calculation of protein–protein ratios.

RESULTS

Expression of p65 and RelB mRNA and protein in human MO, MAC, and DC. Expression of mRNA for Rel/NF-κB family members was analyzed in RNA from freshly isolated MO, *in vitro* differentiated MAC or *in vitro* differentiated DC using RT-PCR. Expression of p65 mRNA was found both in unstimulated MO, MAC, and DC, short-term stimulated MO, MAC and DC and cells stimulated overnight with LPS. p65 mRNA expression was confirmed in independent experiments by Northern blot analysis (Fig. 1). Significant upregulation of the p65 mRNA could be observed in DC and in MAC stimulated overnight with LPS.

For RelB we also found constitutive mRNA expression in unstimulated MO, MAC and DC (Fig. 2) by Northern blot analysis. Stimulation with LPS for 30 min did not result in upregulation of RelB mRNA expression in MO, MAC, or in DC. In freshly isolated MO RelB mRNA was even downregulated after LPS stimulation, although this effect was variable in different experiments with individual donors. Overnight stimulation of MO and MAC failed to increase RelB transcription (Fig. 2). In contrast, RelB mRNA was significantly upregulated in DC stimulated by LPS overnight, leading to a four- to fivefold increase compared to unstimulated DC (Fig. 2).

When we analyzed whole cell extracts (WCE) of either MO, MAC, or DC by Western blotting we could detect p65 and RelB protein in all three cell types with an especially high RelB protein content in stimulated DC (data not shown).

Analysis of NF-κB binding activity and characterization of the different NF-κB subunits. Although the presence of NF-κB proteins is a prerequisite for their action as transcription factors, part of their biological activity is regulated by their subcellular localization. Therefore we determined the NF-κB binding activity in nuclear preparations of human MO, MAC, and DC by EMSAs using a ^{32}P-labeled oligonucleotide with an NF-κB binding site from the MHC promoter as a probe. Polyclonal antisera were used in EMSAs to identify individual NF-κB proteins participating in the formation of the different complexes. Antibodies against p105/p50, p52, p65, and RelB were added to the binding reaction subsequent to addition of the radioactively labeled oligonucleotide.

In nuclear extracts from freshly isolated, unstimulated MO we observed one major and two minor complexes (Fig. 3A), indicating the presence of NF-κB binding activity in the nuclei of unstimulated cells. An additional nonspecific complex appeared predominantly in MO and much weaker in the other cell types under study. Complex one was partially supershifted by anti-p50 antibody, the second complex disappeared completely in the presence of anti-RelB antibody and complex three was supershifted by anti-p65 antibody (Fig. 3A). No visible shift was induced by anti-p52 antibody. Thus, it can be assumed that complex one contains p50/p50 homodimers, complex two p50/RelB heterodimers and complex three p50/p65 heterodimers.

EMSAs and supershift experiments with nuclear extracts of stimulated MO showed the same binding activities as described above (data of supershifts not shown). Stimulation of MO with LPS for 30 min re-

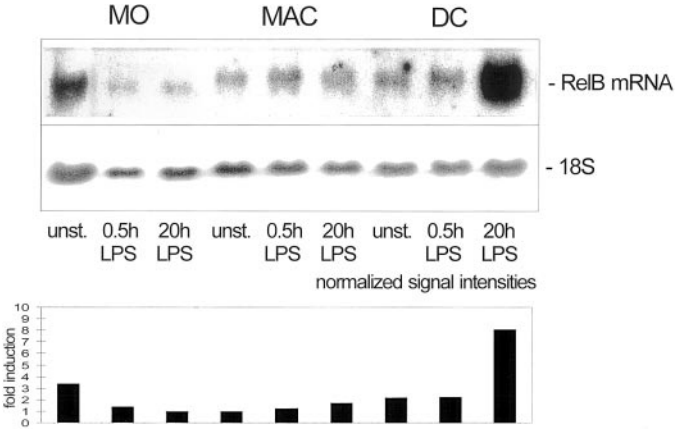

FIG. 2. Expression of RelB mRNA in unstimulated and stimulated MO, MAC, and DC. Northern blot analysis was performed as described in the legend to Fig. 1, but hybridized with a RelB probe. One representative experiment out of three is shown.

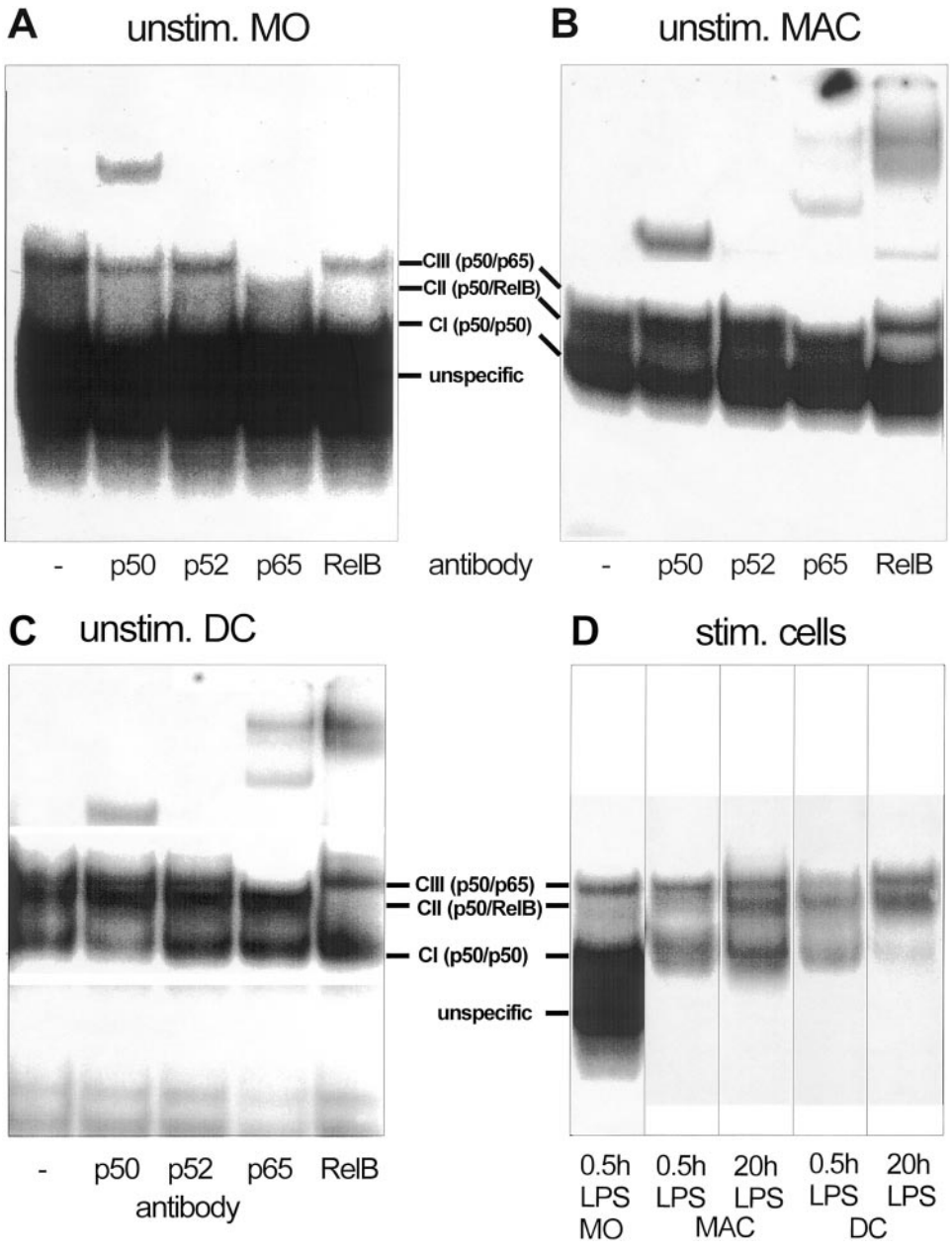

FIG. 3. DNA binding assays with nuclear extracts from MO, MAC, and DC. DNA binding assays were carried out with nuclear extracts of (A) unstimulated MO, (B) unstimulated MAC, (C) unstimulated DC, and (D) stimulated cells. The different NF-κB homo- and heterodimers bound to DNA were identified by supershift experiments as shown in A, B, and C, antibodies being indicated below the figures. Autoradiographs with longer exposure times of EMSAs with MAC and DC nuclear extracts are shown in E. The ratio of bound proteins in the different cell types is shown in F. Experiments were repeated four times with nuclear extracts of different MO donors with similar results; one representative experiment is shown.

sulted in a slight increase in the intensity of the p50/p65 complex (Fig. 3D).

Binding of nuclear proteins from MAC and DC led to different banding patterns in EMSA: while MO, MAC and DC demonstrated common p50/p50, p50/RelB and p50/p65 dimers, MAC and DC exhibited a fourth high molecular weight complex that was absent in MO and present with faint intensity in MAC and DC (Fig. 3E shows the same film as seen in Fig. 3B and Fig. 3C, with an extended film exposure time to demonstrate the appearance of the additional bands). This fourth complex was not affected by antibodies against p50, p52 or RelB, but was completely supershifted by the anti-p65 antibody, indicating the presence of a p65/p65 homodimer.

Whereas, with the exception of p65/p65 described above, the same bands could be identified in unstimulated and stimulated MO, MAC, and DC, the relative

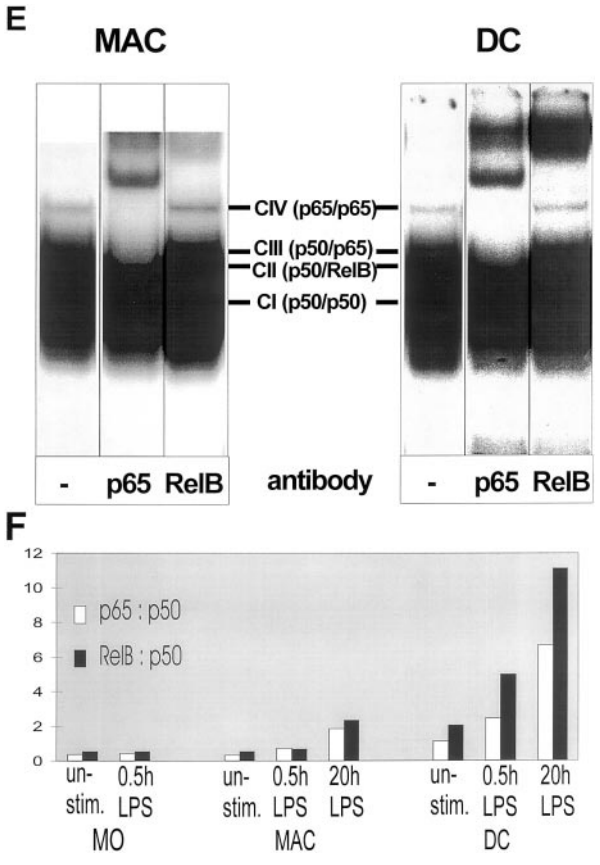

FIG. 3—*Continued*

binding properties of the different dimers differed considerably. In all three cell types, the binding activity of the p50/p50 homodimer was stronger in unstimulated cells compared to cells stimulated by LPS (Fig. 3F). Furthermore, binding of p50/p65 and p50/RelB was considerably upregulated by long term differentiation of MO into MAC or DC. The combined effects of differentiation and stimulation were analyzed by densitometry. Band intensities of the heterodimers were calculated relative to that of p50/p50 (Fig. 3F). Identity of the EMSA bands with regards to specific NF-κB subunits was analyzed by antibody dependent supershift experiments as described above (detailed data of all analyses are not shown). The relative binding intensity of the heterodimers was strongest during differentiation into DC and after additional stimulation by LPS leading to terminal DC differentiation. In DCs, RelB binding activity was especially prominent (Fig. 3F).

DISCUSSION

Human blood MO, MAC, and DC play a key role in the non-specific immune system and at the bridge to specific immunity (25). Along the differentiation of pluripotent stem cells into cells of the monocytic lineage various transcription factors have been identified which are involved in one or more differentiation stages leading to MO/MAC [reviewed in (26)], but less is known about DC differentiation.

Our findings show constitutive expression of p65 and RelB mRNA in freshly isolated, nonadherent, unstimulated MO and in contrast to data from de Wit *et al.* (27) we found no significant upregulation after short-term LPS stimulation of MO by Northern blot analysis. Furthermore we found both p65 and RelB protein constitutively expressed in MO and could clearly demonstrate by EMSA that the expressed protein also has DNA-binding activity in the nucleus. Several authors (28, 29) similarly report the presence of low levels of p50/p65 binding activity in the nuclei of unstimulated MO. In contrast, Conti *et al.* (30) found no p65 protein in MO and a rapid turnover of p65 mRNA in MO cultured for one day. Pettit *et al.* (31) reported the absence of RelB protein in freshly isolated unstimulated MO. These contradictory results may be due to a family of serine proteases exclusively expressed in MO, which are able to degrade NF-κB proteins, as shown for p65 (32). Special precautions should be taken when preparing protein extracts from MO.

When we analyzed NF-κB expression and DNA binding activity after differentiation of MO into either MAC or DC, we found only slight upregulation of p65 and RelB expression on the mRNA and protein levels. However, when binding activities of p50/p65 and p50/RelB were compared to that of p50 homodimers, a very strong increase in the ratio of the binding activities was observed during differentiation into DC and an even stronger increase during the differentiation into MAC. Further upregulation was induced after stimulation by LPS especially for RelB in terminally differentiated DC. The data are summarized in Table 1.

Conti *et al.* (30) showed an increase in mRNA expression and DNA binding activity of NF-κB in MAC compared to MO, but only p50 and p65 were examined. Pettit *et al.* (31) stated that RelB mRNA is absent in human MO, but can be induced following stimulation of MO with LPS for 18 h and RelB binding activity is thereupon induced during DC differentiation. The data in these papers are partially confirmatory to our results describing an upregulation of p65/p65 in MAC and an upregulation of p50/RelB in DC. However, in both papers the specific NF-κB member under examination was not detected in freshly isolated MO, indicating an 'all-or-none' result. We assume that we probably searched for the respective proteins under more stringent conditions and therefore observed gradual instead of absolute differences.

To our knowledge, this study represents the first direct comparison of the different NF-κB family members during differentiation of MO into either MAC or DC. Based on previous work by other groups we had expected an upregulation of p65 after stimulation with

TABLE 1

mRNA Expression, Protein Expression, and DNA Binding Activity of NF-κB in MO, MAC, and DC

		MO[a]			MAC			DC		
Treatment		Unstim	0.5 h LPS	20 h LPS	Unstim	0.5 h LPS	20 h LPS	Unstim	0.5 h LPS	20 h LPS
mRNA expression[a]	p65	+[c]	+	+	+	+	++	++	++	+++
	RelB	+	+	+	+	+	+	+	+	++++
Protein expression[d]	p65	+	+	++	++	++	++	++	++	++
	RelB	+	+	++	+	+	++	+	+	++++
DNA binding activity[e]	p65	+	++	nd[f]	++	++	+++	++	++	+++
	RelB	+	+	nd	++	++	+++	++	++	++++

[a] MO, MAC, and DC (unstimulated/stimulated) were obtained as described under Materials and Methods.
[b] mRNA data were generated by Northern blot analysis.
[c] Expression was determined by densitometric evaluation: +, weak expression; ++, medium expression; +++, strong expression; ++++, very strong expression.
[d] Protein expression was analyzed by Western blotting.
[e] DNA binding activity data were obtained by EMSA.
[f] nd, not done.

LPS and after MAC differentiation (27, 30), whereas an upregulation of RelB in DC seemed plausible. The parallel increases in p50/RelB, p50/p65 and p65/p65 binding activities during MAC and DC differentiation are perhaps unexpected in the light of the mouse knock out studies. However, although the shifts in the expression and binding activity are slight, they are probably biologically important. It has already been described by others that binding of p50/p50 homodimers may silence gene expression (33) and therefore the increases in p50/p65 and p50/RelB binding activities compared to that of p50/p50 may indicate increased transcriptional activity in both MAC and DC. However, a considerable upregulation of RelB at mRNA, protein and DNA binding activity levels is seen in DC additionally stimulated by LPS. It is known, that stimulation of DC by LPS or other agents induces their terminal differentiation from a state of antigen uptake into a state of antigen presentation and T cell stimulation [reviewed in (34)]. This terminal differentiation step of DC clearly correlates to the prominent expression of RelB, although p65 is also upregulated. We assume, that RelB expression is especially important for this terminal maturation step. Accordingly, in RelB knockout mice, the number of Langerhans cells is normal, but the number of antigen-presenting thymic DC is significantly reduced (18). For a precise understanding of this fact, a more detailed knowledge about NF-κB target genes that are especially important for DC function is necessary. The various NF-κB dimers display different optimal binding affinities for the multiple-κB DNA motifs (35), but RelB binding characteristics were never included in a study on this topic. Furthermore, the biological effects of different NF-κB dimers binding to the same DNA motif are not yet clear. Further experiments are needed to understand which DC specific versus MAC specific genes are under transcriptional control of the individual NF-κB dimers using different promoter sequences. Data from knockout models and the different expression and DNA binding pattern of individual NF-κB family members in MO derived cell types suggests an important role of NF-κB in the transcriptional control of lineage specific genes and induction of the differentiation program of the respective cell types. Quantitative differences in the activity of NF-κB proteins rather than their absence or presence are probably sufficient and biologically important, as suggested by the data presented above.

ACKNOWLEDGMENTS

The helpful discussions and the critical reading of the manuscript by Dr. M. Rehli and Sven Heinz are gratefully acknowledged.

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