

# A Critical Role for p53 in the Control of NF- $\kappa$ B-Dependent Gene Expression in TLR4-Stimulated Dendritic Cells Exposed to Genistein<sup>1</sup>

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Considerable research has focused on the anti-inflammatory and antiproliferative activities exhibited by the soy isoflavone genistein. We previously demonstrated that genistein suppresses TNF- $\alpha$ -induced NF- $\kappa$ B-dependent IL-6 gene expression in cancer cells by interfering with the mitogen- and stress-activated protein kinase 1 activation pathway. However, effects of isoflavones on immune cells, such as dendritic cells, remain largely unknown. Here we show that genistein markedly reduces IL-6 cytokine production and transcription in LPS-stimulated human monocyte-derived dendritic cells. More particularly, we observe that genistein inhibits IL-6 gene expression by modulating the transcription factor NF- $\kappa$ B. Examination of NF- $\kappa$ B-related events downstream of TLR4 demonstrates that genistein affects NF- $\kappa$ B subcellular localization and DNA binding, although we observe only a minor inhibitory impact of genistein on the classical LPS-induced signaling steps. Interestingly, we find that genistein significantly increases p53 protein levels. We also show that overexpression of p53 in TLR4/MD2 HEK293T cells blocks LPS-induced NF- $\kappa$ B-dependent gene transcription, indicating the occurrence of functional cross-talk between p53 and NF- $\kappa$ B. Moreover, analysis of IL-6 mRNA levels in bone marrow-derived p53 null vs wild-type dendritic cells confirms a role for p53 in the reduction of NF- $\kappa$ B-dependent gene expression, mediated by genistein. *The Journal of Immunology*, 2007, 178: 5048–5057.

Genistein belongs to the category of phenolic nonsteroidal isoflavonoids, which can mainly be found in soybeans and other plants of the Leguminosae family (1). Based on its phytoestrogen characteristic, genistein is pharmaceutically recommended as a dietary supplement for use in alternative hormone replacement therapy to relieve menopausal symptoms (2). Besides that, genistein behaves like an inhibitor of several enzymes or kinases (topoisomerase II, cAMP phosphodiesterase, Akt, tyrosine protein kinase), as an antioxidant due to its phenolic nature, and it affects a host of other intracellular processes (3). We have previously studied the molecular effects of genistein on the TNF- $\alpha$ -stimulated signal transduction toward IL-6 transcription in L2929sA fibroblasts and have shown that genistein affects the kinase cascade toward mitogen- and stress-activated protein kinase 1 (MSK1) activation, which accounts for NF- $\kappa$ B p65 transactivation

via serine 276 phosphorylation and for histone H3 serine 10 phosphorylation on the IL-6 promoter (4). Other research groups have demonstrated that isoflavones have inhibitory effects on classical NF- $\kappa$ B activation (5, 6) and subsequently on NF- $\kappa$ B-dependent antiapoptotic and inflammatory processes, which may contribute to their anticarcinogenic and anti-inflammatory properties. Potential inhibitory effects of genistein on the acquired immune system have been reported in some rodent studies, but the data are inconsistent and even conflicting (7). However, the role of genistein in modulating normal primary cells of the innate immune system and in particular its effect on dendritic cells (DC),<sup>3</sup> have received little attention.

It is well established that DCs, as professional APCs of the innate immune system, reside at the host-pathogen interface and play a key role in directing adaptive immune responses by initializing T cell activity. When immature DCs are triggered by microbial compounds such as LPS, the expression of Ag-presenting MHC class II, accessory molecules and cytokines is up-regulated. Furthermore, depending on the DC maturation status and on the type of cytokines produced by the DC and by the environmental innate immune cells, naive CD4<sup>+</sup> Th cells are instructed to differentiate into Th1, Th2 effector cells or regulatory T cells (8). The role of the prototype cytokines IL-12 and IL-4 in promoting respectively Th1- and Th2-polarized immune responses is well established (9, 10).

However, DC-derived IL-6 is postulated to influence the nature of the immune response too, as this cytokine induces the initial IL-4 production by naive T cells (11, 12). Even more recently is

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Received for publication September 26, 2006. Accepted for publication February 6, 2007.

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<sup>1</sup> This work was supported by Interuniversitaire Attractiepolen p5/12 and the Geconcerteerde Onderzoeksacties. N.D. is a fellow with the Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen. S.Go., S.Ge, and S.F. are postdoctoral researchers of the Fonds National de la Recherche Scientifique, V.A. is supported by Interuniversity Attraction Pole of the Belgian Federal Science Policy, and S.G. and W.V.B. are postdoctoral fellows with the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen.

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; MoDC, monocyte-derived DC; BMDC, bone marrow-derived DC; IKK, I $\kappa$ B kinase; h, human; HEK, human embryonic kidney; fw, forward; rv, reverse; REA, restriction enzyme accessibility assay; gDNA, genomic DNA; poly(IC), polyinosinic-polycytidylic acid; MSK1, mitogen- and stress-activated protein kinase 1; RNAi, RNA interference.

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demonstrated that IL-6 shifts the TGF- $\beta$ -driven regulatory T cell generation into pathogenic T<sub>H</sub>17 cell differentiation (13). Apart from this, IL-6 is involved in a myriad of cancer-, inflammation-, and immunity-related events, because this pleiotropic cytokine induces tumor growth in an autocrine manner, synthesis of acute phase response proteins in hepatocytes, growth of hemopoietic stem cells, and terminal differentiation of B cells into plasma cells and monocytes into macrophages (14) and directs the transition from innate to acquired immunity (15). Therefore, aberrant IL-6 expression has been associated with various chronic inflammatory disorders and autoimmune diseases, such as rheumatoid arthritis, Crohn's disease, psoriasis, etc. (16, 17).

The proinflammatory gene transcriptional program of DCs challenged with LPS is activated through TLR4-induced signal transduction (18), that targets various downstream effectors such as NF- $\kappa$ B. This transcription factor is a dimer composed of Rel protein family members. p52 and p50 precursor molecules (respectively, p100 and p105) and several Rel proteins with a transactivation domain such as p65, c-Rel, and RelB belong to this family. The prototype NF- $\kappa$ B heterodimer, p50-p65, is kept inactive through binding to its inhibitor I $\kappa$ B $\alpha$ . The TLR4-signaling pathway operates via 2 adaptor-dependent mechanisms, which converge by recruitment of TNFR-associated factor 6 and TGF- $\beta$ -activated protein kinase 1. The latter is responsible for MEK, MAPK kinase 4/7, and MAPK kinase 3/6 (and, respectively, ERK, JNK, and p38) activation and, moreover, indirectly activates the I $\kappa$ B kinase (IKK) complex (reviewed in Ref. 19). Subsequently, this complex phosphorylates I $\kappa$ B $\alpha$  on serines 32 and 36 causing its ubiquitinylation and proteasomal degradation. This elimination liberates NF- $\kappa$ B and permits its translocation into the nucleus, where it can bind to  $\kappa$ B promoter elements and induce related gene transcription of cytokines, chemokines, etc. (20).

In this study, we show that LPS-induced IL-6 production by human monocyte-derived DCs (MoDCs) is profoundly down-regulated by genistein. However, unlike previous data obtained on TNF- $\alpha$ -treated fibroblast cancer cells, MAPK signaling is only slightly affected, suggesting that genistein acts through different mechanisms in DCs. We have therefore analyzed the impact of this phytochemical on NF- $\kappa$ B activation and localization. Furthermore, we have explored potential p53-NF- $\kappa$ B cross-talk in response to genistein, which may be responsible for down-regulation of NF- $\kappa$ B-dependent gene expression. On the whole, our results suggest that genistein acts as a potent modulator of DC functions and these findings highlight the anti-inflammatory and immunomodulatory properties of genistein, because DCs are critical players in the initiation and regulation of immune responses. In this view, genistein may represent an attractive dietary tool to dampen unwanted cellular immune response and excessive cytokine production after transplantation or in autoimmune diseases.

## Materials and Methods

### Mice

p53<sup>-/-</sup> and p53<sup>+/+</sup> mice on a C57/BL6 background were described by Jacks et al. (21). Female and male mice were used for bone marrow extraction at the age of 4–7 wk. The mice were bred and maintained in specific pathogen-free conditions according to the institutional guidelines.

### Generation of DCs

MoDCs were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 20  $\mu$ g/ml geomyacin, 1% nonessential amino acids, and 50  $\mu$ M 2-ME. MoDCs were generated from PBMCs from healthy donors. Briefly, PBMCs in medium were allowed to adhere onto 75-cm<sup>2</sup> flasks. After 2 h at 37°C, nonadherent cells were removed; and after extensive washing, adherent cells were cultured in complete medium containing recombinant human (h) GM-CSF

(800 U/ml; Schering-Plough) and hIL-4 (200 U/ml; R&D Systems). Every 2 days, 800 U of hGM-CSF and 200 U of hIL-4 were added. On day 6 of culture, nonadherent cells, which correspond to the MoDC-enriched fraction, were harvested and used for experiments.

Bone marrow DCs (BMDCs) were cultured in RPMI 1640 Glutamax (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 55  $\mu$ M 2-ME, and nonessential amino acids (Invitrogen). BMDCs were flushed with sterile PBS from the femurs and tibias of mice; 3.5  $\times$  10<sup>6</sup> cells per 10-cm dish were plated out in complete medium supplemented with 10 ng/ml murine recombinant GM-CSF (Biosource and Peprotech). New medium with murine GM-CSF was added on day 3, and primary culture was replated on day 7 similarly to methods for day 0, followed by addition of fresh medium with murine GM-CSF on day 10. Cells were used in subsequent experiments on day 12. The BMDC population obtained with this protocol (originally described by Lutz et al. in Ref. 22) contained routinely >70% of CD11c<sup>+</sup> DCs as assessed by FACS.

### Stable cell lines, plasmid constructs, and reagents

The human embryonic kidney (HEK)293T cells stably expressing TLR4/MD2 have been described previously (23). The luciferase reporter constructs p1168hu.IL6P-luc<sup>+</sup> and p(IL6- $\kappa$ B)<sub>3</sub>50hu.IL6P-luc<sup>+</sup> have been used before (24, 25). The expression vector pCMV-HA-p53 and RNA interference (RNAi) construct pSUPER-p53 were described by Unger et al. (26) and Brummelkamp et al. (27), respectively. The pRcRSV-p65 expression vector was described earlier (28). LPS from *Salmonella enterica* (serotype *abortus equi*, used for HEK293T and BMDC) and from *Escherichia coli* (serotype 0128:B12, used for MoDC), DMSO, and genistein were from Sigma-Aldrich. Both LPS types were dissolved in sterile water and used at a final concentration of 1  $\mu$ g/ml. FSL1 and polyinosinic-polycytidylic acid (poly(IC)) are from InvivoGen and Amersham, respectively. Genistein was dissolved in DMSO to a stock concentration of 80 mM. DMSO was used as solvent control in an equal volume to the highest concentration of genistein treatment (0.25% v/v unless differently indicated in the figure legends). In none of the experiments did DMSO treatment show significant effects. Therefore, no-solvent conditions or DMSO controls were not shown in some experiments.

### Transient transfection and luciferase assays

HEK293T cells were seeded in 24-well dishes and transiently transfected using the Lipofectamine transfection methods according to the manufacturer's instructions (Invitrogen). After 18 h, cells were treated as indicated. Promoter activities were analyzed as described elsewhere (24).

### EMSA

Nuclear and cytoplasmic extracts were prepared as described previously (25). Following quantification of protein amounts by the Bradford assay, 10  $\mu$ g of nuclear extracts were analyzed for their binding activity to an IL-6-derived  $\kappa$ B sequence-containing probe essentially as described previously (29). The NF- $\kappa$ B oligonucleotide 5'-AGCTATGTGGGATTTTC C C ATGAGC-3' was labeled with Klenow enzyme using [ $\alpha$ -<sup>32</sup>P]dCTP, and electrophoresis was conducted on a 6% native polyacrylamide gel. For supershift assays, anti-p65 C20 and anti-p50 NLS were included in the reaction mixture. The gels were dried and exposed to phosphorimager screens, which were scanned by StormScan Phosphorimager (Molecular Dynamics).

### Western blotting

Total cellular extracts were made in Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT). Equal amounts of cytoplasmic/nuclear extracts or equal volumes of total lysates from each condition were resolved by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and analyzed by Western blotting. Chemiluminescent detection was performed using HRP-coupled secondary Abs and Western lightning chemiluminescent reagent plus (PerkinElmer Life Sciences) on the Kodak image station 440CF. Anti-P-IKK, anti-P-ERK, anti-P-p38, anti-P-JNK, and anti-P-MSK1 were obtained from Cell Signaling. Anti-I $\kappa$ B $\alpha$  C21, anti-PARP H250, anti-Grb2 C23, anti-p53 DO1, anti-p65 C20, and anti-p50 C19 were purchased from Santa Cruz Biotechnology.

### Flow cytometry

DCs were analyzed for the expression of cell surface molecules by flow cytometry. The following mouse anti-human IgG1 fluorochrome-coupled Abs were used: CD80-PE, CD86-PE, HLA-DR-FITC, and CD40-FITC (BD Biosciences).

Table 1. Genistein decreases IL-6 protein levels in stimulated MoDCs<sup>a</sup>

| Conditions      | IL-6 (pg/ml) |                |
|-----------------|--------------|----------------|
|                 | Donor 1      | Donor 2        |
| Untreated       | 38.5         | 49.0           |
| DMSO            | 41.5         | 37.0           |
| Gen             | 19.0         | Not detectable |
| LPS             | 44,500.0     | 39,125.0       |
| LPS + DMSO      | 43,250.0     | 36,656.3       |
| LPS + Gen       | 2281.3       | 3000.0         |
| Poly(IC)        | 5326.1       | 844.9          |
| Poly(IC) + DMSO | 5467.4       | 867.0          |
| Poly(IC) + Gen  | 398.8        | 47.4           |
| FSL1            | 2456.5       | 2690.6         |
| FSL1 + DMSO     | 2608.7       | 2921.9         |
| FSL1 + Gen      | 212.5        | 103.8          |

<sup>a</sup> DCs were either treated or not treated with genistein (Gen; 200  $\mu$ M) or DMSO for 1 h and then stimulated with LPS (1  $\mu$ g/ml), poly(IC) (10  $\mu$ g/ml), or FSL1 (100 ng/ml) for 6 h. IL-6 protein levels in culture supernatants were analyzed by ELISA. Data represent absolute values from two donors.

### Quantification of cytokine production

All cytokine levels in cell-free culture supernatants were determined using specific ELISA kits (Biosource) with detection limits of 15 pg/ml, according to the manufacturer's instructions.

### RNA isolation and RT-PCR

Total RNA was extracted with the acid guanidinium thiocyanate-phenol-chloroform method using the Trizol reagent (Invitrogen). Reverse transcription was performed on 0.5  $\mu$ g of total RNA in a 30- $\mu$ l total volume. After 1/5 dilution, quantitative real time PCR was performed on 5  $\mu$ l of each condition using Bio-Rad iQ Supermix (for probe assay) or Invitrogen Sybr green platinum Supermix-UDG on a iCycler apparatus (Bio-Rad). The probe and primer sets were designed by primer3 software: hIL-6 forward (fw), GACAGCCACTC ACCTCTCA; hIL-6 reverse (rv), AGTGCCTCTTTGCTGCTTTC; hIL-6 probe, (6-FAM)CCTCGACGGCATCTCAGCCC(TAMRA) (phosphate); h- $\beta$ -actin fw, GGATGCAGAAGGAGATCACTG; h- $\beta$ -actin rv, CGATC CACACGGAGTACTTG; h- $\beta$ -actin probe, (6-Fam)CCCTGGCACCCAG CACAATG(Tamra)(phosphate); mL-6 fw, GAGGATACCACTCCCAA CAGACC; mL-6 rv, AAGTGCATCATCGTTGTTCATACA.

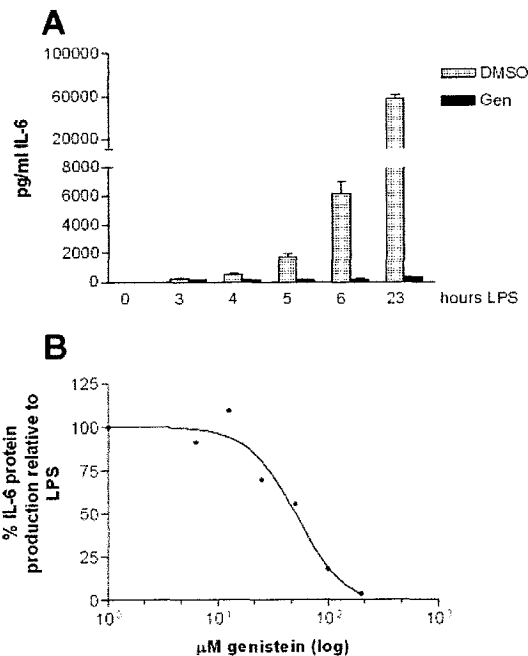
A serial dilution of a cDNA mix standard was used to determine the efficiency of the PCR. All amplifications were performed in duplicate or triplicate, and data were analyzed using Genex software (Bio-Rad) taking primer set efficiency into account.

### Immunofluorescence assay

DCs were fixed with 2% paraformaldehyde-PBS, washed in PBS, and stored in methanol at  $-20^{\circ}\text{C}$ . For the staining, DCs were attached to cytoslides by cytospin in PBS or to coverslips and permeabilized with 0.1% Triton X-100 followed by blocking with 2% BSA. For MoDC, coverslips were then incubated with rabbit polyclonal NF- $\kappa$ B p65 Ab A (Santa Cruz Biotechnology) for 1 h at room temperature; after extensive washing, samples were incubated with the anti-rabbit Alexa 568 from Molecular Probes (Invitrogen). Then the slides were mounted in Vectashield mounting medium (Vector Laboratories). Specimens were analyzed with the Leica confocal SP2 laser scanning microscope. For BMDCs, cytoslides were incubated overnight with rabbit polyclonal p65 Ab C20 (Santa Cruz Biotechnology) at  $4^{\circ}\text{C}$ , washed, and incubated with anti-rabbit Alexa 488 (Molecular Probes and Invitrogen). Nuclei were stained using 4',6-diamidino-2-phenylindole, and coverslips were mounted on cytoslides with Vectashield (Vector Laboratories). Analysis was performed on the Zeiss Axiovert 200M immunofluorescence microscope.

### Restriction enzyme accessibility assay (REA)

The REA technique was performed essentially as described earlier (30) with some modifications. In brief, nuclei were extracted using buffer A (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.3 M sucrose) supplemented with 1 mM sodium butyrate and 0.5% Nonidet P-40. Purified nuclei ( $5 \times 10^6$ ) in buffer A with 1 mM sodium butyrate, 100  $\mu$ g/ml BSA, and 0.1 mM PMSF were partially digested by 40 U of *NheI* (Promega) or *BsrBI* (New England Biolabs) restriction enzymes for 30 min at  $37^{\circ}\text{C}$ . Reactions were stopped by addition of  $2\times$  proteinase K buffer (100 mM



**FIGURE 1.** Genistein (Gen) decreases IL-6 production in LPS-stimulated MoDCs. DCs were left untreated, treated with genistein (200  $\mu$ M or as indicated) or DMSO for 1–2 h, and then stimulated with LPS (1  $\mu$ g/ml) as indicated (A) or during 24 h (B). IL-6 cytokine concentration was measured in the supernatants by ELISA. A, Time kinetics data are expressed as concentration IL-6 in picograms per milliliter. B, A 1/2 dilution series of genistein from 200  $\mu$ M to 6.25  $\mu$ M final concentration was made. ELISA results are expressed as percentage of IL-6 induction relative to the LPS condition. A sigmoidal dose-response curve is used for curve fitting. The calculated IC<sub>50</sub> is 52.07  $\mu$ M ( $R^2 = 0.9731$ ). The LPS + DMSO value (110%) is not included in the graph.

Tris-HCl (pH 7.5), 1% SDS, 200 mM NaCl, 2 mM EDTA). After proteinase K and RNase A treatment, genomic DNA (gDNA) was twice phenol-chloroform and once chloroform extracted and dissolved in sterile water after ethanol precipitation. Purified gDNA (7–15  $\mu$ g) was digested overnight with 30 U of *PstI* enzyme (Promega). Samples were analyzed by electrophoresis on a 1.5% agarose gel. After denaturation of the gel, capillary transfer to Hybond N<sup>+</sup> membrane (Amersham) and UV-cross-linking, hybridization was performed with a <sup>32</sup>P-labeled probe (*HindIII-PstI*) spanning nt +1756 to nt +2448 from the hIL-6 gene. The obtained bands *NheI-PstI* and *BsrBI-PstI* are 2674 and 2565/3021 bp, respectively. The restriction enzyme sites and band lengths are based on the human IL-6 mRNA sequence with accession number NM\_000600, blasted to the human genome on www.ensembl.org.

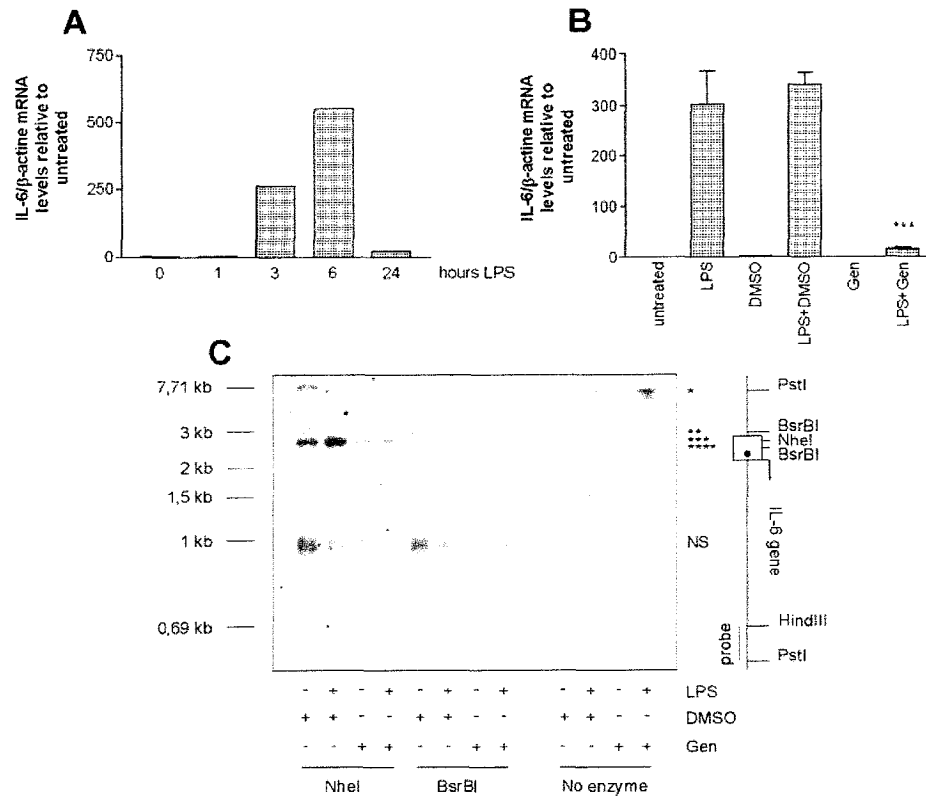
### Statistics

All statistical calculations were done in Graphprism version 3.0, according to one-way ANOVA (Bonferroni's multiple comparison test) except for the data of Fig. 7B, which has been analyzed using an unpaired two-tailed Student *t* test. The degree of significance is indicated in the figures by: \*,  $p < 0.5$ ; \*\*,  $p < 0.1$ ; and \*\*\*,  $p < 0.01$ .

## Results

### Genistein strongly down-regulates TLR-dependent IL-6 production in MoDCs

In a first series of experiments, we examined whether or not genistein influences IL-6 cytokine production in MoDCs, in response to TLR2, TLR3, and TLR4 agonists. Investigation of IL-6 levels in the supernatant samples of two donors shows increased secreted IL-6 protein amounts after FSL1, poly(IC) and LPS stimulation, which are significantly reduced when pretreated with genistein (Table I). Because LPS elicits the highest IL-6 response,



**FIGURE 2.** Genistein (Gen) represses IL-6 transcription in LPS-stimulated MoDCs. **A**, DCs were stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ) for different times. IL-6 mRNA levels were normalized to  $\beta$ -actin mRNA values. **B**, DCs were left untreated, pretreated with genistein (200  $\mu\text{M}$ ) or DMSO for 1 h, and stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ) during 6 h. IL-6 mRNA results were normalized to  $\beta$ -actin mRNA levels, and the mean values of four different donors were calculated ( $\pm$ SD). The results are expressed relatively to the control setup (untreated). \*\*\*, vs LPS or LPS + DMSO. The LPS value is not significantly different from the LPS + DMSO value. **C**, DCs were pretreated with genistein (200  $\mu\text{M}$ ) or DMSO for 2 h and then stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ) during 3 h. REA was performed as described in *Materials and Methods*. After treatment, DCs of four different donors were pooled per treatment condition. Of each, 15  $\mu\text{g}$  of control and *BsrBI*-treated genomic DNA and 7  $\mu\text{g}$  of *NheI*-treated samples were used for Southern blotting. Control samples of each condition were included in the experiment and treated totally similarly, except for the in vivo enzyme digestion. One representative experiment of three is shown. \*, *PstI-PstI* band, 7710 bp; \*\*, *BsrBI-PstI* band, 3021 bp; \*\*\*, *NheI-PstI* band, 2674 bp; \*\*\*\*, *BsrBI-PstI* band, 2565 bp; NS, nonspecific band. The IL-6 gDNA is visualized by a straight line, on which the position of the IL-6 proximal promoter and the NF- $\kappa$ B site are indicated by an open rectangle and a filled circle, respectively. The probe, restriction enzyme cutting sites, and transcriptional start position are also shown.

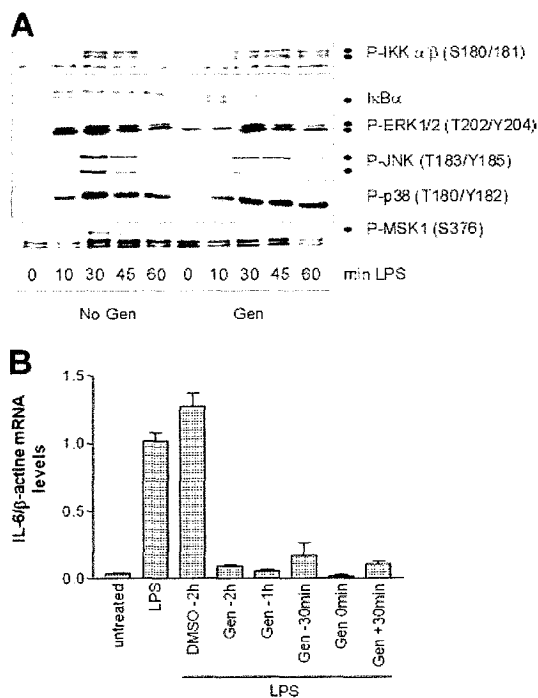
we further focused on this stimulus. A time kinetics experiment with LPS with/without genistein pretreatment reveals that LPS stimulation increases IL-6 levels and that genistein has a potent inhibitory effect on IL-6 cytokine production (Fig. 1A). An additional dose-response experiment, covering genistein concentrations from 200  $\mu\text{M}$  to 6.25  $\mu\text{M}$  confirms its repressive effect on LPS-stimulated IL-6 levels (Fig. 1B). A dose of 200  $\mu\text{M}$  genistein, which was shown not to affect MoDC viability after 6 h of treatment, as assessed by phosphatidylinositol-annexin V-Alexa Fluor 488 staining (data not shown), was used in all subsequent experiments.

#### Genistein significantly reduces LPS-stimulated IL-6 transcription in MoDCs

To verify whether genistein affects IL-6 at the (post)translational level or acts at the transcriptional level, we performed quantitative real time RT-PCR on IL-6 mRNA. A time kinetics experiment with LPS was performed to examine the rate and extent of IL-6 mRNA production in MoDCs (Fig. 2A). This assay shows detectable IL-6 mRNA amounts already after 1 h, clearly abundant levels after 3 h, which rise up to 6 h or later. At 24 h, IL-6 signals are almost basal again. mRNA analysis after 6 h of LPS stimulation shows that IL-6

levels of MoDCs pretreated with genistein are strongly reduced (Fig. 2B), whereas the  $\beta$ -actin mRNA levels remain unaffected.

To strengthen the indications that genistein negatively affects IL-6 mRNA production, we performed a restriction enzyme accessibility assay on the IL-6 gene promoter in MoDC as a read-out for chromatin relaxation and transcriptional activity (Ref. 31 and M. N. Ndlovu, C. Van Lint, D. Chalbos, G. Haegeman, and W. Vanden Berghe, submitted for publication). By the use of selected restriction enzymes that cut on specific positions in the IL-6 promoter, the local chromatin accessibility can be unveiled. Because the chromatin configuration and level of chromatin opening are tightly controlled and related to transcription initiation in general (32–35), this test allows us to investigate possible effects of genistein on IL-6 transcription. As shown in Fig. 2C, after 3 h of LPS treatment, we can clearly observe a higher accessibility in the IL-6 proximal promoter region of MoDCs, as revealed by *NheI* or *BsrBI* digestion. In contrast, genistein pretreatment efficiently abrogates the LPS-stimulated chromatin opening. A more upstream promoter region containing an additional recognition site for the *BsrBI* enzyme was not remodeled upon LPS stimulation. The corresponding 3021-bp fragments can be considered as an internal control, showing equal loading of gDNA.

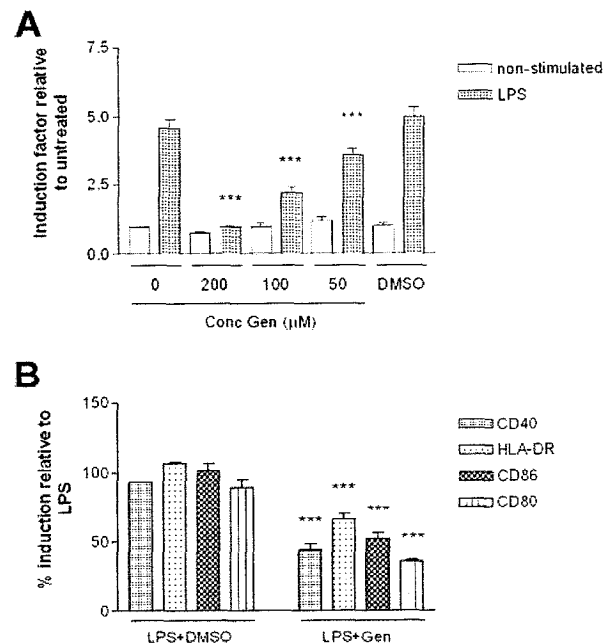


**FIGURE 3.** TLR4-dependent signaling cascades toward IL-6 gene transcription in MoDCs are not affected by genistein (Gen). *A*, DCs were either or not pretreated with genistein (200  $\mu$ M) for 2 h, followed by stimulation with LPS (1  $\mu$ g/ml) for the indicated times. Total cellular lysates were prepared, blotted, and analyzed with the respective (phospho-specific) Abs to visualize I $\kappa$ B $\alpha$  degradation and the amount of phosphorylated (P-) kinases in a time kinetics. The occurrence of aspecific bands and the control with anti-p65 for the different membranes (not shown) show equal protein loading in each lane. The presented pattern is a representative result of Western blotting experiments on a minimum of two donors. *B*, DCs were left untreated or treated with genistein (200  $\mu$ M) or DMSO as indicated: 2 h, 1 h, 30 min before, simultaneously, or 30 min after adding LPS (1  $\mu$ g/ml). The LPS stimulation lasted for 3 h. IL-6 mRNA levels were normalized to  $\beta$ -actin mRNA values.

*Proximal TLR4-initiated kinase signaling pathways in MoDCs are not significantly influenced by genistein*

The clear reduction of IL-6 transcription roused us to explore whether genistein affects the related molecular signaling cascade toward the IL-6 gene promoter. MoDCs were either or not pretreated with genistein and stimulated with LPS in a 10- to 60-min kinetics (Fig. 3*A*). Western analysis of lysates shows LPS-induced IKK, MAPK (ERK, p38, JNK), and MSK1 activation in a time-dependent manner, with peak phosphorylations at 30 min. Consequently, the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  is degraded at this time point and is even not resynthesized at 60 min. In contrast to the profound effects in fibroblast L929sA cells (4), genistein treatment only slightly lowers the phosphorylation peaks of MAPKs and MSK1, but does not affect the IKK phosphorylation pattern. However, statistical analysis of the band intensities of at least 2 Western blots (by Image J software) reveals that the observed differences at either time point do not reach significance (data not shown).

In line with the lack of effect on proximal receptor signaling, we still observe a significant reduction of IL-6 mRNA levels if genistein is added 30 min after LPS stimulation instead of 1–2 h in advance (Fig. 3*B*).



**FIGURE 4.** NF- $\kappa$ B-dependent promoter activity in TLR4/MD2 HEK293T cells is dose-dependently reduced by genistein (Gen). *A*, TLR4/MD2 HEK293T cells were transiently transfected with the p(IL6- $\kappa$ B)<sub>3</sub>50hu.IL6P+ reporter construct. Inductions were performed in triplicate. Cells were left untreated or treated with genistein (200, 100, 50  $\mu$ M) or DMSO during 2 h and stimulated overnight with LPS (1  $\mu$ g/ml). Equal amounts of protein lysates were analyzed in luciferase assays, and values are expressed as fold induction ( $\pm$ SD) relatively to the control (untreated). Two other independent experiments gave similar results. \*\*\*, vs LPS or LPS + DMSO. The LPS value is not significantly different from the LPS + DMSO value. *B*, DCs were either or not pretreated with genistein (200  $\mu$ M) for 1 h and then stimulated with LPS (1  $\mu$ g/ml) during 12 h. Expression of immunostimulatory molecules for DC function (CD40, HLA-DR, CD80, CD86) was analyzed by flow cytometry. DMSO vehicle control-treated cells did not show significant differences with LPS-treated cells. Results are plotted as percentage of expression relative to LPS values and are the mean ( $\pm$ SD) of three different donors, using mean values of histograms. \*\*\*, vs LPS + DMSO. Conc., Concentration.

*NF- $\kappa$ B-dependent promoter activity in TLR4/MD2 HEK293T cells is significantly reduced by genistein*

Because genistein potently inhibits TLR4-mediated IL-6 gene expression without profoundly affecting upstream signaling events such as MAPK and IKK activation, we continued by investigating further downstream events of these signal cascades. We performed reporter studies on a HEK293T cell line, which stably expresses TLR4/MD2 proteins (23). Of all known transcription factors binding to the IL-6 promoter region, we focused on NF- $\kappa$ B because of its crucial role in triggering IL-6 transcription (36) and also because genistein and other isoflavones were previously reported to inhibit NF- $\kappa$ B-mediated processes (5, 6, 37–40). In accordance with the dose-dependent effect of genistein on endogenous IL-6 levels in MoDCs, genistein significantly lowers the LPS-stimulated NF- $\kappa$ B-dependent promoter activity in these HEK293T cells (Fig. 4*A*). Moreover, in line with the results obtained with the synthetic NF- $\kappa$ B reporter construct containing multimerized  $\kappa$ B sites, a similar dose-dependent genistein repression could be observed with another reporter construct, driven by the endogenous hIL-6 promoter sequence (results not shown). Furthermore, a severely impaired LPS response was measured in reporter experiments with a NF- $\kappa$ B-mutated IL-6 promoter construct (data not shown).