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Novel Regulatory Mechanisms of CD40-Induced Prostanoid Synthesis by IL-4 and IL-10 in Human Monocytes

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Interleukins IL-4 and IL-10 are considered to be central regulators for the limitation and eventual termination of inflammatory responses in vivo, based on their potent anti-inflammatory effects toward LPS-stimulated monocytes/macrophages and neutrophils. However, their role in T cell-dependent inflammatory responses has not been fully elucidated. In this study, we investigated the effects of both cytokines on the production of PGE₂, a key molecule of various inflammatory conditions, in CD40-stimulated human peripheral blood monocytes. CD40 ligation of monocytes induced the synthesis of a significant amount of PGE₂ via inducible expression of the cyclooxygenase (COX)-2 gene. Both IL-10 and IL-4 significantly inhibited PGE₂ production and COX-2 expression in CD40-stimulated monocytes. Using specific inhibitors for extracellular signal-related kinase (ERK) and p38 mitogen-activated protein kinase (MAPK), we found that both kinase pathways are involved in CD40-induced COX-2 expression. CD40 ligation also resulted in the activation of NF-κB. Additional experiments exhibited that CD40 clearly induced the activation of the upstream kinases MAPK/ERK kinase 1/2, MAPK kinase 3/6, and IκB in monocytes. IL-10 significantly inhibited CD40-induced activation of the ERK, p38 MAPK, and NF-κB pathways; however, inhibition by IL-4 was limited to the ERK pathway in monocytes. Neither IL-10 nor IL-4 affected the recruitment of TNFR-associated factors 2 and 3 to CD40 in monocytes. Collectively, IL-10 and IL-4 use novel regulatory mechanisms for CD40-induced prostanoid synthesis in monocytes, thus suggesting a potential role for these cytokines in regulating T cell-induced inflammatory responses, including autoimmune diseases. The Journal of Immunology, 2004, 172: 2147–2154.
originally described as soluble factors preferentially synthesized in Th2 cells (19, 20), appear to be prototypic anti-inflammatory cytokines that limit inflammatory responses. Many investigators, including us, have previously shown that both cytokines are capable of inhibiting the expression of a wide range of proinflammatory cytokines in LPS-stimulated monocytes/macrophages (17, 18, 21). We have previously demonstrated that IL-10 and IL-4 significantly blocked LPS-induced PGE2 production via down-regulation of COX-2 induction in monocytes (17, 18, 22). The significant role of IL-10 in the regulation of prostanoids in vivo was further shown by other authors using IL-10-deficient mice (23). However, these studies were mostly performed using LPS-stimulated cells. Therefore, we cannot simply deduce from these reports that IL-10 and IL-4 are central regulators under different inflammatory conditions in vivo. Given the previous observations that IL-10 and IL-4 co-operatively alleviate the progression of arthritis models in mice (24–26), both cytokines might exert a potent regulatory effect on the T cell-dependent inflammatory responses.

In the present study, we first investigated prostanoid synthesis in monocytes following CD40 ligation, and its regulation by IL-10 and IL-4. Furthermore, we took these observations one step further and investigated the underlying molecular mechanisms.

Materials and Methods

Reagents

LPS was purchased from Difco (Detroit, MI). NS-398, a specific inhibitor of COX-2, was purchased from Alexis (San Diego, CA). PD98059, a specific inhibitor of MAPK/ERK kinase 1 (MEK1), was purchased from Cell Signaling Technology (Beverly, MA). SB203580, a specific inhibitor of p38 MAPK, was kindly provided by SmithKline Beecham Pharmaceuticals (King of Prussia, PA). Human rIL-10 was kindly provided by K. Moore (Plough, Parsippany, NJ). Human rIL-10 was kindly provided by Plough (Bloomfield, NJ). Soluble CD40L (sCD40L) was obtained from Alexis. Rabbit polyclonal Abs against control or phospho-specific ERK1/2, MEK1/2, p38 MAPK, MAPK kinase 3/6 (MKK3/6), and I-κB were purchased from Cell Signaling Technology. Curcumin was obtained from Sigma (St. Louis, MO). An anti-COX-2 mAb was purchased from Transduction Laboratories (Lexington, KY). An anti-human CD40 mAb (BE-1) was purchased from Ancell (Minneapolis, MN). Anti-human TNFR-associated factor 2 (TRAF2) (H-249) and anti-human TRAF3 polyclonal Abs (H-122) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation and culture of human monocytes

Human monocytes were isolated and cultured, as previously described (17, 18, 27). Buffy coats from healthy donors were provided by the Fukuoka Red Cross Blood Center (Fukuoka, Japan). Briefly, PBMCs were separated by Ficoll-Hypaque method. Subsequently, monocytes were isolated from human monocytes (5 × 10⁵ cells) obtained from informed healthy volunteers were treated with IL-4 or IL-10 for 2 h before stimulation with 100 ng/ml sCD40L. The cells were collected at 18 h after stimulation, in which we observed the maximal prostanoid production in monocytes (data not shown).

Enzyme immunoassay (EIA) for PGE2, thromboxane B2 (TXB2), and leukotriene B4 (LTB4) and ELISA for TNF-α

Determinations of PGE2, TXB2, LTB4, and TNF-α levels in the culture supernatants of monocytes (3 × 10⁵ cells) were conducted using commercially available kits for PGE2, TXB2, LTB4 EIA (Cayman Chemicals, Ann Arbor, MI), and TNF-α ELISA (BioSource International, Camarillo, CA), respectively. The culture supernatants were collected at 24 h after stimulation, in which we observed the maximal prostanoid production in monocytes (data not shown).

Analysis of COX-2 protein expression

Monocytes (4 × 10⁵ cells) were treated at 37°C with or without PD98059, SB203580, curcumin, IL-4, or IL-10 for 2 h before stimulation with 100 ng/ml sCD40L. The cells were collected at 18 h after stimulation, in which we observed the maximal COX-2 expression in monocytes (data not shown). The cell pellets were lysed in 50 μl of SDS sample buffer (62.5 mM of Tris-HCl, pH 6.8, 10% glycerol, 2% (v/v) SDS, 50 mM of DTT, 0.1% (v/v) BAP at 25°C. The cell lysates were then sonicated for 15 s and centrifuged at 15,000 × g for 15 min. The resulting cell lysates (2 × 10⁶ cells/lane) were separated by 12.5% SDS-PAGE and transferred onto a polyvinylidine difluoride membrane. The membrane was blocked with PBS containing 5% skim milk and 0.1% Tween 20, and then incubated with 0.25 μg/ml mouse anti-COX-2 mAb at 25°C for 2 h. The membrane was subsequently incubated with a peroxidase-linked species-specific Fab’2, from anti-mouse sheep Ig (1/1000 dilution), and analyzed using the Amersham ECL system (Amersham, Arlington Heights, IL).

Analysis of MAPK phosphorylation

Monocytes (4 × 10⁵ cells/lane) were treated at 37°C with or without IL-4 or IL-10 for 2 h before stimulation with 100 ng/ml sCD40L. After various time intervals, the cell pellets were lysed on ice for 30 min with 40 μl of lysis buffer (1% Triton X-100, 50 mM of HEPES, pH 7.4, 150 μM of sodium pyrophosphate, 50 mM of NaF, 1 mM of Na3VO4, 1 mM of PMSF). After centrifugation, supernatants were subsequently mixed with an equal amount of 2× SDS sample buffer. The resulting cell lysates (2 × 10⁶ cells/lane) were subjected to Western blot analysis using rabbit polyclonal control or phospho-specific MAPK (ERK1/2, p38 MAPK, MEK1/2, MKK3/6) Ab (1/1000 dilution). The membrane was subsequently incubated with a peroxidase-linked species-specific Fab’2, from anti-rabbit donkey Ig (1/2000 dilution), and analyzed using the Amersham ECL system.

Immunoprecipitation

The procedure of immunoprecipitation was previously described (27). Monocytes (5 × 10⁵ cells/lane) were treated with IL-4 or IL-10 for 2 h before stimulation with sCD40L for 15 min. The cell pellets were lysed in the lysis buffer (1% Triton X-100, 50 mM of Tris-HCl, pH 7.5, 150 mM of NaCl, 5 mM of EDTA, 10 mM of NaF, 2 mM of Na3VO4, 2 mM of PMSF, 0.05 mg/ml aprotinin, 2 mM of N-ethylmaleimide), and cell lysates were then immunoprecipitated with 30 μl of protein G plus protein A-agarose preconjugated (Oncogene Research Products, San Diego, CA) and 2 μg of anti-human CD40 mAb overnight at 4°C. The beads were washed four times with buffer A and resuspended with 1/5 SDS sample volume. From 5 × 10⁶ monocytes were subjected to Western blot analysis using anti-TRAF2 Ab, anti-TRAF3 Ab, or anti-human CD40 mAb (1/1000 dilution). The membrane was subsequently incubated with a peroxidase-linked species-specific Fab’2, from anti-rabbit donkey Ig (1/2000 dilution) or anti-mouse donkey Ig (1/2000 dilution), and analyzed using the Amersham ECL system.

Extraction of nuclear proteins and EMSA

The procedures for extraction of nuclear proteins and EMSA have previously been described (27). Briefly, human monocytes (5 × 10⁵ cells) were treated at 37°C with or without IL-4 or IL-10 for 2 h before stimulation by 100 ng/ml sCD40L and then lysed to extract nuclear proteins. Nuclear extracts (2 μg) were mixed with binding buffer (20 mM of HEPES-NaOH, pH 7.9, 2 mM of EDTA, 100 mM of NaCl, 10% glycerol, 0.2% Nonidet P-40), poly(dI-dC), and a 32P-labeled oligonucleotide probe. The mixtures were incubated at room temperature for 30 min. The reaction mixtures were loaded onto a 4% polyacrylamide gel and electrophoresed with 0.25× Tris base, boric acid, EDTA (TBE) running buffer. The gel was then dried onto Whatman 3MM paper (Whatman, Clifton, NJ). The DNAprotein complex was visualized by autoradiography. The sequence of the oligonucleotide probe used to detect the DNA-binding activities of NF-κB was: NF-κB, 5’-GCT CAT GGG TTT CTC CAC CAA G-3’ (28) (the NF-κB binding site is underlined).

Isolation of total RNA from human monocytes and RT-PCR assay

Monocytes (5 × 10⁵ cells) obtained from informed healthy volunteers were treated at 37°C with or without IL-4 or IL-10 for 2 h before stimulation by 100 ng/ml sCD40L. After stimulation for 6 h, total RNA from 5 × 10⁶ monocytes was extracted by using the guanidinium isothiocyanate/phenol extraction method (Isogen; Nippon Gene, Tokyo, Japan), quantified by measuring the absorbance at 260 nm, and stored at −80°C until further analysis. RNA (1 μg) preparation was used for first-strand cDNA synthesis (RNA PCR kit; PE Biosystems, Urayasu, Japan). The reaction was performed according to the manufacturer’s recommendations. Briefly, the reverse transcriptase reaction was conducted at 42°C for 15 min and the
reacted was terminated by heating at 99°C for 5 min, followed by rapid chilling on ice.

**Primer design for competitive PCR assay and generation of DNA competitor**

The specific primers used are as follows: for COX-2 (29), 5'-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3' (forward), 5'-AGA TCA TCT CTG CCT GAG TAT CCT-3' (reverse), predicted size of fragment 301 bp; for GAPDH (30), 5'-CCA TGG AGA AGG CTG GGG-3' (forward), 5'-CAA AGT TGT CAT GGA TCA GC-3' (reverse), predicted size of fragment 195 bp. Composite primers were engineered to contain sequences that amplified the cDNA fragment with the gene-specific primer sequences flanking their 5' ends. The DNA competitors were designed so that the PCR product from the cDNA could be separated from that of its competitor and were generated using reagents supplied in a commercial kit (Comprehensive DNA Construction Kit, Takara Shuzo, Otsu, Japan). Briefly, a 30-cycle PCR was conducted on cDNA using the corresponding primers for the target sequence. Concentrations of the DNA competitors were then measured using a spectrophotometer and adjusted so that each stock concentration was 2 × 10^5 copies/μl.

**Competitive PCR assay**

A series of competitive PCR was set up using 3- or 10-fold dilutions of the DNA competitors with a constant amount of the first-strand cDNA and performed in 20-μl PCR mixtures. PCR was performed using a program of 30 cycles (GAPDH) or 35 cycles (COX-2) of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final 10-min extension at 72°C. The amplified products were subjected to electrophoresis on a 3% agarose gel.

**Quantitative real-time PCR and Taqman probes and primers**

The primers and probes used in the real-time PCR of COX-1, COX-2, and GAPDH mRNA were designed using the Primer Express program and synthesized (Applied Biosystems, Tokyo, Japan) as follows: for COX-1, 5'-CAGAGACCCAACAGCAGTGATG-3' (forward), 5'-GCGCCCAATACG (reverse), and 5'-FAM-TTCCTACCACCAGCAACCCTGCCA-TAMRA-3' (probe); for COX-2, 5'-GAATCATTCACC (forward), 5'-FAM-TTCTTACACCCAGCAACTTCGCA-TAMRA-3' (reverse), and 5'-FAM-AGGTGAAGGTCGGAGTCCCTGTGCACCA-3' (probe). Concentrations of the DNA competitors were then measured using a spectrophotometer and adjusted so that each stock concentration was 2 × 10^5 copies/μl.

**Results**

First, we tested whether CD40 ligation could induce PGE2 production by monocytes. We found that unstimulated monocytes constitutively produced a small amount of PGE2, while CD40-stimulated monocytes produced a dramatically larger amount of PGE2, which is known to be synthesized by COX-2 (Fig. 1A). Although neither IL-4 nor IL-10 affected the basal PGE2 production by monocytes, both cytokines showed the similar significant (p < 0.01) inhibition of CD40-induced PGE2 production by monocytes, as assessed in Fig. 1A. In addition, CD40 induced the generation of TXB2, the other COX-2-dependent product, which was again inhibited by IL-4 and IL-10 (Fig. 1B). In contrast, the production of LTβR, a COX-2-independent product, was not affected by CD40, IL-4, or IL-10 (Fig. 1C).

Next, we examined whether or not CD40-induced PGE2 production depended on CD40, a crucial enzyme catalyzing a limiting step of prostaglandin synthesis. As shown in Fig. 2, NS-398, a specific COX-2 inhibitor, significantly (p < 0.05) blocked CD40-induced, but not constitutive (data not shown), PGE2 production by monocytes in a dose-dependent manner, suggesting that the COX-2 enzyme is actively involved in this process.

In response to various stimuli, the amount of COX-2 protein is significantly increased in monocytes (14–16). The expression of COX-2 protein in CD40-stimulated monocytes was also markedly induced after 18 h. IL-4 (85% inhibition, p < 0.01) and IL-10 (97% inhibition, p < 0.01) significantly inhibited this expression (Fig. 3).
(Fig. 3). To further confirm whether both cytokines regulated COX-2 expression at the upstream of the protein level, we examined COX-2 mRNA expression using a competitive PCR assay. As shown in Fig. 4 (A and B), the amount of COX-2 mRNA in CD40-stimulated monocytes was ~30-fold more (6 × 10^5 copies) than that in unstimulated monocytes (2 × 10^4 copies). Notably, COX-2 mRNA expression was inhibited by IL-4 (6 × 10^4 copies) and IL-10 (2 × 10^4 copies). These findings were further confirmed by using a quantitative real-time PCR (Fig. 4C), CD40 ligation of monocytes induced COX-2 mRNA expression (>21-fold), which was significantly inhibited by IL-4 and IL-10 (IL-4, p < 0.01; IL-10, p < 0.01). We also examined COX-1 mRNA expression; however, we could not quantify it probably due to low expression levels in monocytes even after CD40 ligation (data not shown).

To determine whether MAPK pathways are involved in CD40-induced PGE_2 production and COX-2 expression in monocytes, we tested the effects of specific inhibitors for MEK1/2 (PD98059) and p38 MAPK (SB203580) in CD40-stimulated monocytes. We have previously shown the specificity of these inhibitors in monocytes (18). Both inhibitors significantly (p < 0.01) inhibited PGE_2 production (Fig. 5A) as well as COX-2 protein expression (p < 0.01) (Fig. 5, B and C), suggesting that the ERK and p38 MAPK pathways are involved in CD40-induced prostanooid synthesis.

We next examined the effects of IL-4 and IL-10 on the activation of the ERK and p38 MAPK pathways in CD40-stimulated monocytes. CD40 ligation caused significant ERK phosphorylation, which was inhibited by IL-4 (79% inhibition, p < 0.01) and IL-10 (84% inhibition, p < 0.01) (Fig. 6A). CD40 ligation also led to the phosphorylation of MEK1/2, an upstream kinase required for ERK phosphorylation. MEK1/2 phosphorylation was also inhibited by IL-4 (79% inhibition, p < 0.05) and IL-10 (90% inhibition, p < 0.01) (Fig. 6B). Moreover, CD40 ligation induced phosphorylation of p38 MAPK and MKK3/6, an upstream kinase required for p38 MAPK phosphorylation (Fig. 6, C and D). IL-10 partially, but significantly inhibited the phosphorylation of p38 MAPK and MKK3/6 (60%, p < 0.05 and 49%, p < 0.05), while IL-4 had no effect on either kinase (Fig. 6, C and D). These results suggest that IL-10 regulates CD40-induced activation of the ERK and p38 MAPK pathways, while IL-4 regulates that of the ERK, but not the p38 MAPK pathway.

To date, there have been several reports demonstrating that CD40, like other TNFR family members, not only induces MAPK activation, but also induces NF-κB activation. We thus investigated whether the NF-κB pathway is involved in CD40-induced PGE_2 production in monocytes by using an inhibitor of NF-κB, curcumin (31, 32). PGE_2 production in monocytes was significantly (p < 0.01) inhibited in a dose-dependent manner, and complete inhibition was achieved in the presence of 50 μM of curcumin (Fig. 7). We next examined the effects of IL-4 and IL-10 on CD40-induced NF-κB activation in monocytes using an EMSA.

**FIGURE 3.** Effects of IL-4 and IL-10 on CD40-induced COX-2 protein expression. Human monocytes (4 × 10^5 cells) were treated for 2 h with or without IL-4 (10 ng/ml) or IL-10 (10 ng/ml), and then stimulated with sCD40L (100 ng/ml) for 18 h. After stimulation, cell lysates (2 × 10^6 cells/lane) were collected and used to determine the expression of COX-2 protein by Western blot analysis (A). The densitometric analyses were conducted from three separate experiments, and the mean (±SD) fold inductions compared with control samples are indicated (B). **, p < 0.01 (compared with CD40 only).

**FIGURE 4.** IL-4 and IL-10 regulate CD40-induced COX-2 mRNA expression in monocytes. Human monocytes (5 × 10^5 cells) from healthy donors were treated for 2 h with or without IL-4 (10 ng/ml) or IL-10 (10 ng/ml), and then stimulated for 6 h with sCD40L (100 ng/ml). After stimulation, total RNA was extracted and then first-strand cDNA was synthesized by RT-PCR. A, To quantify the amount of COX-2 mRNA, each sample was diluted to be adjusted by quantifying the amount of total mRNA by competitive PCR using the internal standard (GAPDH) competitor. Finally, competitive PCR for COX-2 was performed using serial 3-fold dilutions of competitors (indicated), and the amplified products were subjected to electrophoresis on a 3% agarose gel. Larger sized bands are PCR products derived from competitive fragments, of which concentrations are indicated above the bands. B, The fold induction compared with a control, analyzed using a competitive PCR, is indicated. C, COX-2 mRNA expression analyzed using a real-time PCR. The relative mRNA amounts of COX-2 vs GAPDH are expressed as the mean ± SD of triplicate samples. Similar results were obtained in three separate experiments. **, p < 0.01 (compared with CD40 only).
IL-4 and IL-10 exert differential effects on the NF-κB inhibitory effect (Fig. 8). Collectively, these results suggested that B cells (2 x 10^6) stimulated for 18 h with sCD40L (100 ng/ml) and then cultured for 24 h with or without 100 ng/ml sCD40L. The concentrations of these inhibitors were determined to give a maximal inhibition of activation of kinases and be less influential to cell viability in our preliminary experiments (data not shown). Culture supernatants were assayed for PGE2 by EIA (A). Results are expressed as the mean ± SD of triplicate cultures. Monocytes (4 x 10^6 cells/well) were treated for 2 h with or without IL-4 (10 ng/ml) or IL-10 (10 ng/ml), and then stimulated for 18 h with sCD40L (100 ng/ml). After stimulation, cell lysates (2 x 10^6 cells/lane) were collected and used to determine the expression of COX-2 protein by Western blot analysis (B). The densitometric analyses were conducted based on three separate experiments, and the mean (±SD) fold inductions compared with control samples are indicated (C). **p < 0.01 (compared with CD40 only).

CD40 ligation resulted in strong NF-κB activation in monocytes. IL-10 significantly (p < 0.05) blocked this NF-κB activation, whereas IL-4 showed little suppressive effect (Fig. 8A). Consistent with the EMSA data above, I-κB was markedly phosphorylated after CD40 ligation, and IL-10 inhibited its phosphorylation (70% inhibition, p < 0.05). However, IL-4 failed to show such an inhibitory effect (Fig. 8B). Collectively, these results suggested that IL-4 and IL-10 exert differential effects on the NF-κB pathway, namely IL-10 inhibited CD40-induced NF-κB activation in monocytes partly via the inhibitory effect on I-κB phosphorylation.

Finally, we examined whether IL-4 or IL-10 affects the recruitment of TRAF proteins to CD40 in monocytes (Fig. 9). A previous study showed that TRAF2 and TRAF3 are recruited to CD40 in B cells upon CD40 ligation (33). Before stimulation, both TRAF2 and TRAF3 associated with CD40 to some extent in monocytes. CD40 ligation facilitated further recruitment of TRAF2 and TRAF3 to CD40 at 15 min, in which we observed the maximal TRAF recruitment to CD40 in our preliminary experiments (data not shown). IL-4 and IL-10 had no effect on the recruitment of TRAF2 and TRAF3 to CD40, suggesting that each cytokine regulates CD40 signaling at the downstream levels of TRAF recruitment to CD40.

**Discussion**

Activation of monocytes/macrophages is a crucial event for the development of the inflammatory response in vivo because these cells are capable of synthesizing a wide array of proinflammatory mediators in response to a number of soluble factors such as LPS, cytokines, and mitogens. LPS is the most extensively studied inducer of the synthesis of proinflammatory cytokines and prostanooids in monocytes/macrophages (34, 35). In addition to soluble factors, it is becoming appreciated that monocytes/macrophages can also be strongly activated through direct interaction with other cells, such as activated T cells. In fact, the CD40L-CD40 system appears to be important for this function. Previous studies have shown that CD40 ligation induces the production of proinflammatory cytokines in monocytes (1, 2). In this study, we have shown that CD40 ligation on monocytes significantly induces the synthesis of PGE2, a pivotal proinflammatory mediator. Moreover, this process is dependent on CD40-induced COX-2 expression in monocytes. Consistent with our findings, Zhang et al. and Cao et al. (36, 37) demonstrated that CD40 ligation induced PGE2 production in lung and orbital fibroblasts via the induction of COX-2 expression. Zhang et al. showed that CD40 ligation alone induced only minimal COX-2 mRNA expression, and further up-regulation was achieved by the pretreatment of these cells with IFN-γ. This might be partly due to the up-regulation of CD40 expression on the cell surface by IFN-γ because we have found that the same is true in monocytes in our system (data not shown).

We also showed that IL-10 and IL-4 strongly inhibited CD40-induced PGE2 production in monocytes via down-regulation of COX-2 expression at the gene level. This trend is similar to our observations of LPS-induced COX-2 expression in monocytes, as previously described (17, 18). In contrast, the effects of both cytokines appeared to be different depending on the molecules induced by CD40 ligation. Both cytokines inhibited IL-1 production to a similar extent (38). Interestingly, IL-10 inhibited, but IL-4 enhanced, CD40-induced IL-12 production, while both cytokines significantly blocked LPS/IFN-γ-induced IL-12 production in macrophages (39). We found that IL-10 strongly, but IL-4 only partially, inhibited CD40-induced TNF-α production in monocytes (data not shown). Among a wide range of proinflammatory mediators, prostanoind synthesis might be a common target for regulatory roles of IL-10 and IL-4 in vivo.

In this study, we investigated the underlying molecular mechanisms for prostanoind regulation by IL-10 and IL-4. In particular, we focused on the MAPK and NF-κB pathways because these pathways had been suggested to play a key role in inflammatory responses (40, 41). By using a specific inhibitor of each pathway, it was suggested that the ERK and p38 MAPK pathways are involved in CD40-induced COX-2 expression in monocytes. Subsequent experiments indicated that CD40 ligation induced the phosphorylation of ERK1/2 and its upstream kinase MEK1/2 in monocytes, consistent with recent studies (42, 43). CD40 ligation also induced the phosphorylation of p38 MAPK and its upstream kinase MKK3/6 in monocytes. Similar observations were reported using B and monocyte-derived dendritic cells (44, 45). In contrast to our current findings, Suttles et al. (42) found CD40 ligation induced activation of ERK, but not p38 MAPK in monocytes. This discrepancy might be due to a difference in the means of CD40 stimulation, because plasma membrane products from activated CD4 T cells were used in their study, while the pure soluble CD40 ligand was used in our study (42).

Intriguingly, IL-10 and IL-4 exerted differential effects on the activation of ERK and p38 MAPK in CD40-stimulated monocytes.
Both cytokines significantly inhibited CD40-induced ERK activation, consistent with the previous study (42). In addition, IL-10 and IL-4 inhibited phosphorylation of MEK1/2 to a similar level. In contrast, IL-10, but not IL-4, inhibited activation of p38 MAPK and MKK3/6. These results are in agreement with our previous studies on LPS-stimulated monocytes/macrophages (18). Collectively, these observations strongly suggest that both cytokines differentially regulate the activation of kinases upstream of MEK1/2 and MKK3/6. One of the earliest events in CD40 signaling involves the recruitment of TRAF proteins to CD40 (33). Intriguingly, neither IL-10 nor IL-4 affected the recruitment of TRAF2 and TRAF3 to CD40 in monocytes. This suggests that IL-10 and IL-4 regulate CD40 signaling pathways at the downstream levels of TRAF recruitment to CD40. Further work is still necessary to

**FIGURE 6.** Effects of IL-4 and IL-10 on the activation of the ERK and p38 MAPK pathways in CD40-stimulated monocytes. Human monocytes (4 × 10⁶ cells) were treated for 2 h with or without IL-4 (10 ng/ml) or IL-10 (10 ng/ml), and then stimulated for 1 h (A and B) or 10 min (C and D) with sCD40L (100 ng/ml). After stimulation, cell lysates (2 × 10⁶ cells/lanes) were collected and used to determine the phosphorylation of ERK1/2, MEK1/2, p38MAPK, or MKK3/6 by Western blot analysis. The densitometric analyses were conducted based on three separate experiments, and the mean (±SD) fold inductions compared with control samples are indicated. *, p < 0.05; **, p < 0.01 (compared with CD40 only).

**FIGURE 7.** NF-κB pathway is involved in CD40-induced PGE₂ production in monocytes. Human monocytes (3 × 10⁶ cells/well) were pre-stimulated for 1 h with the indicated concentrations of curcumin, and then cultured for 24 h with or without 100 ng/ml sCD40L. Culture supernatants were assayed for PGE₂ by EIA. Results are expressed as the mean ± SD of triplicate cultures. **, p < 0.01 (compared with sCD40L only).

**FIGURE 8.** Effects of IL-4 and IL-10 on CD40-induced NF-κB activation in monocytes. Human monocytes were treated for 2 h with or without IL-4 (10 ng/ml) or IL-10 (10 ng/ml), and then stimulated for 10 min (A) or 1 h (B) with sCD40L (100 ng/ml). After stimulation, nuclear proteins were extracted and assayed for NF-κB DNA-binding activity by EMSA (A), and cell lysates were collected and used to determine the phosphorylation of IκBα by Western blot analysis (B). The densitometric analyses were conducted based on three separate experiments, and the mean (±SD) fold inductions compared with control samples are indicated (B). *, p < 0.05 (compared with CD40 only).
define which level of CD40 signaling pathways is target for IL-10 and IL-4. STAT3 and STAT6 play a crucial role in the signaling pathways for IL-10 and IL-4, respectively (46, 47). The mechanisms by which STAT activation by these cytokines leads to their inhibitory effects on the MAPK pathways remain to be clarified.

In addition to the MAPK pathway, CD40 ligation eventually leads to NF-κB activation (2, 48, 49). We indicated that the NF-κB pathway is also involved in CD40-induced COX-2 expression in monocytes, and IL-10 remarkably inhibited CD40-induced NF-κB DNA-binding activity, consistent with previous studies using different stimuli (50–52). In addition to the inhibition of IκB phosphorylation, there may be other mechanisms involved in IL-10-mediated inhibition of NF-κB (50). In contrast, IL-4 had a less pronounced inhibitory effect on CD40-induced NF-κB activation and IκB phosphorylation in monocytes in this study. Given that IL-4 partially inhibited CD40-induced TNF-α production in monocytes (data not shown), NF-κB activation might be a more dominant signaling pathway for the control of TNF-α production. It has been shown that IL-4 inhibits NF-κB-dependent gene expression via the activation of STAT6 in macrophages (53–55). Thus, we cannot rule out the possibility that IL-4 might directly regulate the transcriptional activity of NF-κB-regulated genes.

The results in this study strongly support the hypothesis that IL-4 and IL-10 are central regulators of prostanooid synthesis, even when T cell-dependent inflammation is dominant. In light of the fact that CD40L expression is often observed in activated T cells surrounding inflamed lesions in a number of autoimmune disorders, both cytokines seem more likely to provide a beneficial therapeutic application for these conditions. Indeed, both cytokines clearly ameliorate the progression of autoimmune disease models in mice such as RA and experimental autoimmune encephalomyelitis (56–59). It is thus possible that these cytokines may work in concert with suboptimal doses of other anti-inflammatory drugs to produce a superior therapeutic outcome with fewer adverse effects. Obviously, further investigations are necessary to unravel regulatory mechanisms of IL-10 and IL-4, which will help us to develop improved methods of using these cytokines in various clinical situations.

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References


