Increased Nuclear Factor-κB Activation in Peripheral Blood Monocytes of Patients with Rheumatoid Arthritis Is Mediated Primarily by Tumor Necrosis Factor-α

ISABELLE DICHAMP, ALAIN BOURGEOIS, CARINE DIRAND, GEORGES HERBEIN, and DANIEL WENDLING

ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is a disease characterized by prolonged production of tumor necrosis factor-α (TNF-α), which is regulated by the Rel/nuclear factor-κB (NF-κB) transcription factors. We assessed NF-κB activation in peripheral blood mononuclear cells (PBMC), peripheral blood lymphocytes (PBL), and monocytes from patients with RA, patients with ankylosing spondylitis (AS), and healthy subjects.

Methods. NF-κB activation was determined by electrophoretic mobility shift assays and by Western blotting in PBMC, monocytes, and PBL isolated from peripheral blood of patients with RA, patients with AS, and healthy subjects and determined after *ex vivo* pretreatment of PBMC, PBL, and monocytes of patients with RA and healthy subjects with infliximab and with etanercept.

Results. Enhanced NF-kB activation was observed in monocytes, PBL, and PBMC isolated from patients with RA, but not in PBMC, PBL, and monocytes of patients with AS and healthy subjects. The NF-κB complex was composed of p50 and p65 subunits and its activation required inhibitor of NF-κBα degradation. We observed a positive correlation between the NF-κB activation in monocytes, PBL, and PBMC, and TNF-α levels in peripheral blood of patients with RA. *Ex vivo* treatment with infliximab and etanercept decreased NF-κB activation in monocytes of patients with RA, but not in PBL and PBMC, and not in healthy subjects.

Conclusion. Our results indicate a role for NF-κB activation and TNF-α in the activation of monocytes of patients with RA, and suggest an important role of circulating monocytes in RA pathogenesis. (First Release Sept 15 2007; J Rheumatol 2007;34:1976–83)

Key Indexing Terms:
MONOCYTES
RHEUMATOID ARTHRITIS
NUCLEAR FACTOR-κB
TUMOR NECROSIS FACTOR-α

Rheumatoid arthritis (RA) is a complex autoimmune disease with chronic joint inflammation and progressive bone destruction[1]. A common paradigm for the pathogenesis of RA is that the major histocompatibility-restricted T cell activation in response to an unknown joint-derived antigen results in production of autoantibodies and induction of systemic disease. Rheumatoid synovitis then occurs, characterized by the infiltration of inflammatory cells into the synovial compartment and the production of inflammatory mediators, many of which are thought to be regulated by the Rel/nuclear factor-κB (NF-κB) transcription factor family[2-4]. The concept of 2 components of disease development has been hypothesized: one is a systemic immune component, whereas a second involves local synovial cell activation that may be less dependent on a systemic immune response. Although the etiology and pathogenesis of RA are not fully understood, cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), IL-6, and receptor activator of NF-κB ligand (RANKL) are known to be involved in disease progression[2,5-8]. NF-κB is a crucial regulator of these cytokines[2,5-8].

The Rel/NF-κB family of transcription factors are homo- and heterodimeric proteins comprising subunits encoded by a multigene family related to the *c-rel* protooncogene. Five mammalian Rel/NF-κB proteins have been described to date: NF-κB 1 (p50, p105), NF-κB 2 (p52, p100), RelA (p65), RelB, and c-Rel subunits, encoded by the *NF-κB 1*, *NF-κB 2*, *rela*, *relb*, and *c-rel* genes, respectively[9,10]. In unstimulated cells, the majority of Rel/NF-κB dimers are retained in the cytoplasm as an inactive complex bound to inhibitor proteins (inhibitor of NF-κB; IκB). In response to a wide variety of stimuli, IκB proteins are phosphorylated by the IKK complex[11], targeting the IκB for intracellular degradation[12,13]. Rel/NF-κB dimers then translocate to the nucleus and bind to

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decameric DNA sequences (κB elements) required for the transcription of many genes, including cytokines, chemokines, and adhesion molecules[3]. Functional effects of the different Rel/NF-κB dimers may be dependent on binding preferences for particular κB sequences, as well as varying expression profiles of the individual subunits in tissues[14,15]. Rel/NF-κB has been considered a potential target for therapeutic intervention in inflammatory disease[16-18]. Indeed, it has now been realized that at least part of the antiinflammatory effects of widely used drugs, such as dexamethasone and aspirin, is through inhibition of Rel/NF-κB[19-21].

Increasing evidence shows that chronic inflammatory diseases such as RA are caused by prolonged production of proinflammatory cytokines such as TNF[22]. Monocytic cells, and to a lesser extent peripheral blood lymphocytes (PBL), release TNF-α in response to many stimuli, including the Gram-negative bacterial endotoxin, lipopolysaccharide, TNF-α itself, phorbol esters, superantigen, and viral agents, such as human cytomegalovirus and Epstein-Barr virus[23-26]. Sequences in the proximal 172 bp and in the –627 to –487 bp region of the human TNF-α promoter contribute to transcriptional control in monocytic cells[24,27,28]. This region includes putative CRE/ATF, NF-κB, CCAAT/enhancer-binding protein, and Ets binding sites[24,29]. TNF-α has a key role in the pathogenesis of RA and its antagonists, such as etanercept (Enbrel), a TNFR2 immunoglobulin Fc fusion protein, and infliximab (Remicade), a TNF-α-specific monoclonal antibody, can improve the clinical course of RA[1,30-33]. TNF-α has been demonstrated to be involved in the pathogenesis of ankylosing spondylitis (AS), another chronic inflammatory rheumatic disease, and TNF-α-blocking agents have demonstrated their efficacy in the treatment of the disease[34].

The purpose of our study was to assess NF-κB activation in peripheral blood mononuclear cells (PBMC), peripheral blood lymphocytes (PBL), and monocytes isolated from patients with RA. We show that NF-κB activation is increased in PBMC, PBL, and monocytes isolated from patients with RA compared to patients with AS and healthy subjects. In contrast to PBL and PBMC, we found that enhanced NF-κB activation observed in monocytes is mediated primarily through the production of endogenous TNF-α.

MATERIALS AND METHODS

Patients. Seven adult patients with RA according to the criteria of the American College of Rheumatology[35], 6 adult patients with AS fulfilling the modified New York criteria[36], and 7 age-matched healthy subjects were eligible for enrollment at the Besançon University Hospital. Peripheral blood of these individuals was harvested for measurement of NF-κB activation in monocytes, PBL, and PBMC parallel to plasma TNF-α measurement. Informed consent was obtained from all subjects.

Reagents. The antibody against phospho-IκBα (Ser32) was obtained from Cell Signaling Technology (Beverly, MA, USA). The antibodies anti-p50 (C19, catalog no. sc 109X), anti-p65 (AX, catalog no. sc 52X), anti-RelB (C19, catalog no. sc 226X), anti-c-Rel (B6, catalog no. sc 1190X), and anti-p52 (C5, catalog no. sc 6955X) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against ß-actin was obtained from Sigma (St. Louis, MO, USA). The double-stranded oligonucleotides having the AP-1 and NF-κB consensus sequence were obtained from Eurobio, France. TNF-α levels were measured using an ELISA (R&D Systems, Minneapolis, MN, USA).

Primary cells, PBMC, monocytes, and PBL. Human PBMC were prepared from peripheral blood of healthy donors as described[23]. For purified PBL preparation, Ficoll-Hypaque (Pharmacia, Uppsala, Sweden)-isolated PBMC were incubated for 2 h on 2% gelatin-coated plates. Nonadherent cells, > 98% of which were PBL, as assessed by CD45/CD14 detection by flow cytometry analysis (Simultest Leucogate; Becton Dickinson, San Jose, CA, USA), were harvested after Ficoll-Hypaque isolation. Adherent tissue culture differentiated monocytes (> 94% CD14+ by flow cytometric analysis) were cultured in RPMI medium supplemented with 10% (v/v) pooled human serum (kindly provided by the Etablissement Français du Sang Bourgogne Franche-Comté, France), as reported[37]. An *ex vivo* pretreatment of PBMC, PBL, and monocytes of healthy subjects and patients with RA was carried out for 30 min with both a neutralizing anti-TNF-α antibody (infliximab, 10 µg/ml) and a TNFR2 immunoglobulin Fc fusion protein (etanercept, 10 µg/ml).

Electrophoretic mobility shift assay. To measure NF-κB activation, electrophoretic mobility shift assays (EMSA) were carried out as described[37]. Briefly, 20 µg of nuclear extracts prepared from peripheral blood cells (PBMC, monocytes, PBL) were incubated 20 min with 20 fmol of ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide, 5'-TTG TTA CAA **GGG ACT TTC** CGC TG**G GGA CTT TC**C AGG GAG GCG TGG-3' 3'-AAC AAT GTT CCC TGA AAG GCG ACC CCT GAA AGG TCC CTC CGC ACC-5' (bold type indicates NF-κB binding sites; Eurobio, Les Ulis, France). The same amount of protein was loaded and the DNA-protein complex formed was resolved from free oligonucleotide on a 6% native polyacrylamide gel [6% acrylamide-bis (29:1) (BioRad); 5% glycerol; 2.5 mM Tris-acetate, pH 8.3; 19 mM glycine; 0.1 mM EDTA]).

Composition of buffers. Reactionnal buffer for NF-κB: 2 µl of 10× binding buffer, 2.5% glycerol 50%, 5 mM $MgCl_2$, 50 ng/µl of poly-dI-dC, 0.05% of NP-40 1%. 10× binding buffer: 100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5. TBE 10×: 890 mM of Tris base, 890 mM boric acid, 40 ml EDTA 0.5 M, pH 8.

A double-stranded mutated oligonucleotide, 5'-TTG TTA CAA **CTC ACT TTC** CGC TG**C TCA CTT TC**C AGG GAG GCG TGG-3', was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide and a heterologous unlabeled AP-1 oligonucleotide, 5'-CGC TTG A**TG ACT CA**G CCG GAA-3' (bold type indicates AP-1 binding site). The dried gels were visualized and radioactive bands quantified by a Molecular Images System (BioRad, Hercules, CA, USA) using Multianalyst software.

Western blot analysis. Cytoplasmic extracts of primary PBMC, PBL, and monocytes isolated from the peripheral blood of patients were used to examine IκBα phosphorylation and ß-actin expression by Western blot procedure, as described[37].

Statistical analysis. All database entry and statistical analysis was performed using SPSS software (version 9.0; SPSS). Categorical variables were analyzed using the chi-square or Fisher's exact test. Figures show the means of independent experiments and standard deviation.

RESULTS

We investigated the NF-κB activation in PBMC, monocytes, and PBL isolated from the peripheral blood of patients with RA, patients with AS (Table 1), and healthy subjects without treatment and underlying disease or inflammation. We observed an increased NF-κB activation in PBMC of RA patients with moderate disease activity (Disease Activity Score between 3.4 and 5.5), but not in AS patients and healthy donors (Figures 1A, 1B). Among PBMC, NF-κB activation was observed in monocytes, but also in PBL (Figures 1A, 1B).

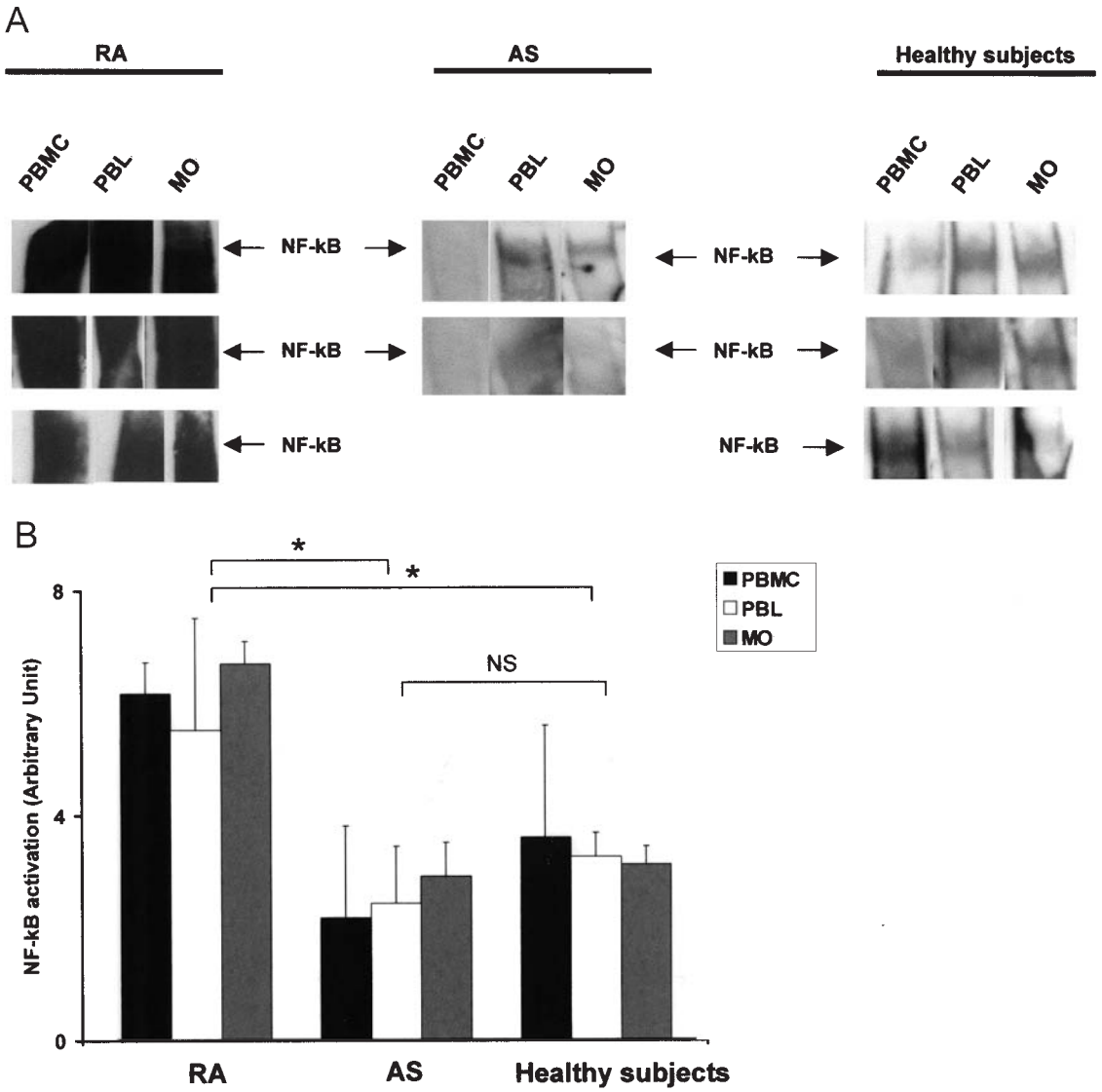

Figure 1. NF-κB activation in PBMC, monocytes (MO), and PBL isolated from patients with RA. A. Nuclear extracts of primary PBMC, monocytes, and PBL (10^7 cells) isolated from patients with RA, patients with AS, and healthy subjects were assayed for NF-κB by EMSA (see Materials and Methods). B. Dried gels were visualized and radioactive bands were quantified. Histogram represents mean values (± SD) of NF-κB activation obtained from 7 patients with RA, 6 with AS, and 6 healthy subjects. The difference between the PBMC, PBL, and monocytes of patients with RA and healthy subjects is significant. *$p < 0.05$.

Supershift analysis with specific antibodies against p65 and p50 indicated that the induced NF-κB activation consisted of p65 and p50, in both monocytes and PBL (Figure 2A and data not shown). Lack of supershift by unrelated antibodies and disappearance of the NF-κB band by competition with unlabeled oligonucleotide indicated that the interaction was specific (Figure 2B). The appearance of the phosphorylated form of IκBα, which is required for IκBα degradation, was examined using Western blot analysis in PBMC, monocytes, and PBL isolated from the peripheral blood of patients with RA. The induction of the phosphorylated form of IκBα was detected in both monocytes and PBL, and was more pronounced in patients with RA than in patients with AS and healthy subjects (Figure 2C and data not shown).

The *ex vivo* pretreatment of monocytes with a neutralizing anti-TNF-α antibody (infliximab 10 μg/ml) significantly diminished NF-κB activation ($p < 0.05$; Figures 3A, 3B). Similarly, *ex vivo* pretreatment of monocytes with a TNFR2 immunoglobulin Fc fusion protein (etanercept 10 μg/ml) significantly decreased NF-κB activation ($p < 0.05$; Figures 3A, 3B). By contrast, no consistent and significant decrease of NF-κB activation was observed in PBMC and PBL of patients with RA following TNF-α blockade (p = nonsignificant; Figures 3A, 3B). Thus, our results indicate that NF-κB activation observed in monocytes isolated from patients with RA is mediated primarily by endogenously produced TNF-α. We did not observe significant changes in NF-κB activation of PBMC, PBL, and monocytes isolated from healthy individu-

Table 1. Data of patients and healthy subjects.

Patient	Age, yrs	Sex	CRP, mg/ml	DAS28	Treatment
1 RA	63	F	41	5.55	LEF + CS
2 RA	75	F	107	5.20	MTX + CS
3 RA	75	F	5	4.67	MTX + CS
4 RA	43	F	5	5.08	MTX + CS
5 RA	53	F	40	4.93	MTX
6 RA	44	F	2	3.43	MTX + CS
7 RA	65	F	4	4.12	MTX + CS
8 AS	58	M	38	—	NSAID + SL
9 AS	26	F	38	—	NSAID + SL
10 AS	45	F	17	—	NSAID
11 AS	56	M	9	—	CS
12 AS	32	M	12	—	NSAID
13 AS	40	M	14	—	NSAID
14 HS	33	M	< 5	—	—
15 HS	24	M	< 5	—	—
16 HS	49	M	< 5	—	—
17 HS	41	F	< 5	—	—
18 HS	52	M	< 5	—	—
19 HS	43	F	< 5	—	—
20 HS	39	M	< 5	—	—

RA: rheumatoid arthritis; AS: ankylosing spondylitis; HS: healthy subject; CRP: C-reactive protein; DAS28: Disease Activity Score (for RA only); SL: sulfasalazine; LEF: leflunomide; MTX: methotrexate; CS: corticosteroids; NSAID: nonsteroidal antiinflammatory drug.

als following TNF-α blockade (p = nonsignificant; Figure 3C). Moreover, a positive correlation between plasma levels of TNF-α and NF-κB activation in monocytes was observed ($r^2 = 0.51$; Figure 4).

DISCUSSION

Both increased TNF-α production and NF-κB activation have been reported to be involved in RA pathogenesis. However, their respective roles in peripheral blood cell subsets have not been studied. We have demonstrated that NF-κB activation is observed in monocytes, PBL, and PBMC isolated from the peripheral blood of patients with RA. We also found that the canonical IKK/p65-p50 pathway is involved in NF-κB activation of PBMC, and that endogenous TNF-α is involved in NF-κB activation of peripheral blood monocytes isolated from patients with RA.

We observed increased NF-κB activation in PBMC of patients with RA. Interestingly, the activation of NF-κB was detected in both monocytes and PBL isolated from the peripheral blood of patients with RA. The level of NF-κB activation was similar in the 2 cell types, further indicating that both monocytes and PBL (including T cells and some B cells) are activated in the peripheral blood of subjects with RA. Our results are in agreement with previous studies indicating that an influx of inflammatory cells such as monocytes, T cells, and B cells from the bloodstream favored the formation of a synovial membrane transformed into a hypertrophic inflammatory tissue[38,39].

TNF-α-induced activation of NF-κB triggers the synthesis and release of proinflammatory cytokines involved in RA, such as TNF-α itself, IL-1, IL-6, monocyte chemoattractant protein-1, and RANKL[40-44]. We observed that the anti-TNF-α monoclonal antibody infliximab, but also the TNFR2 immunoglobulin Fc fusion protein etanercept, significantly diminished NF-κB activation in monocytes, but not in PBL and PBMC, isolated from the peripheral blood of subjects with RA. Thus our results indicate that endogenously produced TNF-α is involved in enhanced activation of NF-κB in peripheral blood monocytes. In addition we found a positive correlation between the levels of plasma TNF-α and NF-κB activation measured within autologous monocytes. Our results are in agreement with a recently published study indicating the amelioration of inflammatory arthritis, such as RA, by targeting the pre-ligand assembly domain of TNF-α receptors[44]. We also observed that the canonical NF-κB pathway (p50/p65) was activated in monocytes and PBL from patients with RA, as reported in primary RA synoviocytes[45-47]. In agreement with this observation, we observed the induction of IκBα phosphorylation in monocytes and PBL of subjects with RA. Since phosphorylation of IκBα at serine is induced, IKK-ß is probably activated, the only kinase known to phosphorylate IκBα directly[48]. In agreement with this hypothesis, NF-κB activation has been reported to be regulated by IKK in primary fibroblast-like synoviocytes[49]. Altogether our results indicate a role for TNF-α in enhanced NF-κB activation observed in peripheral blood monocytes of patients with RA that is mediated by the canonical p50/p65 pathway.

Our results indicate that both infliximab and etanercept significantly diminished NF-κB activation in monocytes, but not in PBL, suggesting a cell-type specificity for the anti-

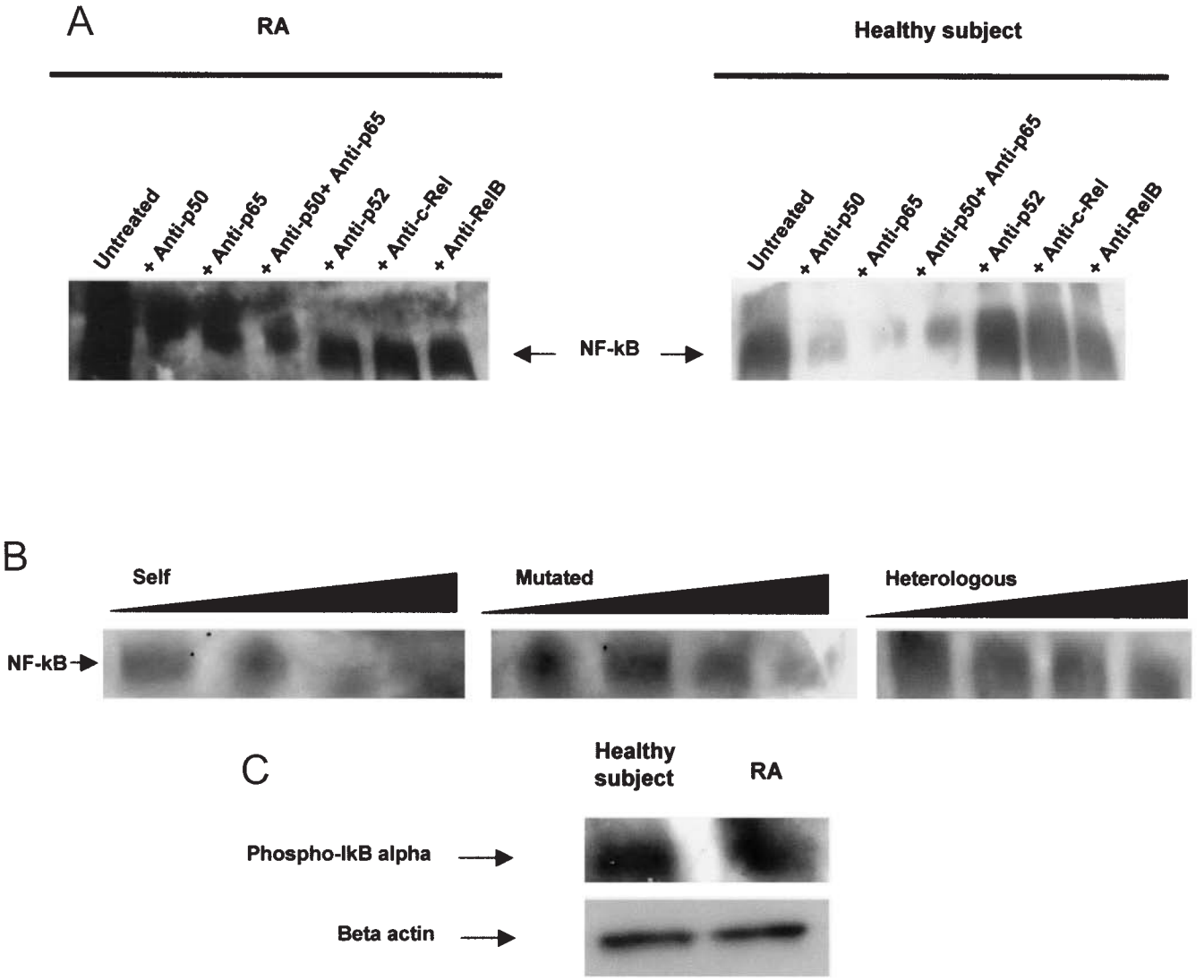

Figure 2. In monocytes from patients with RA, NF-κB complex is composed of p65/p50 heterodimers. A. Interference of NF-κB activation in primary monocytes isolated from peripheral blood of a patient with RA and a healthy control. Nuclear extracts from primary monocytes were incubated 10 min with anti-p50, anti-p65, anti-c-Rel, anti-Rel B, anti-p52, and anti-p50 + anti-p65 antibodies, and then assayed for NF-κB DNA binding activity. B. Specificity of NF-κB activation in primary monocytes isolated from peripheral blood of a patient with RA. Nuclear extracts from primary monocytes were incubated 20 min with increasing concentrations (0, 2, 9, 18 pmol) of unlabeled NF-κB oligonucleotide, unlabeled mutated NF-κB oligonucleotide, and unlabeled heterologous AP-1 oligonucleotide and then assayed for NF-κB DNA binding activity. Data are representative of the 7 patients with RA. C. IκBα phosphorylation in primary monocytes isolated from peripheral blood of a patient with RA and a healthy control. Cytoplasmic extracts from primary monocytes were tested for phosphorylation of IκBα by Western blot. Loading control is shown with ß-actin. Data are representative of 7 patients with RA and 7 healthy subjects.

TNF-α therapy. The effect of infliximab was based on a neutralization of TNF-α and not on a direct cytotoxic/proapoptotic effect of the drug. We did not detect any cell death by trypan blue dye test in the culture following TNF-α blockade (data not shown). Despite the efficacy of agents such as TNF-α inhibitors, a substantial proportion of patients with RA have been reported to have no response[31], to have an unsustained response[50], or to form antibodies against the drugs[51]. In agreement with these observations, among the 7 patients with RA, in one case we did not observe decreased NF-κB activation in monocytes following anti-TNF-α treatment. Altogether, our results indicate that anti-TNF-α treatments decrease NF-κB hyperactivation in monocytes in most patients with RA, and therefore could favor the restoration of peripheral cell-mediated immunity and blockade of the accumulation of inflammatory cells in joints[52,53].

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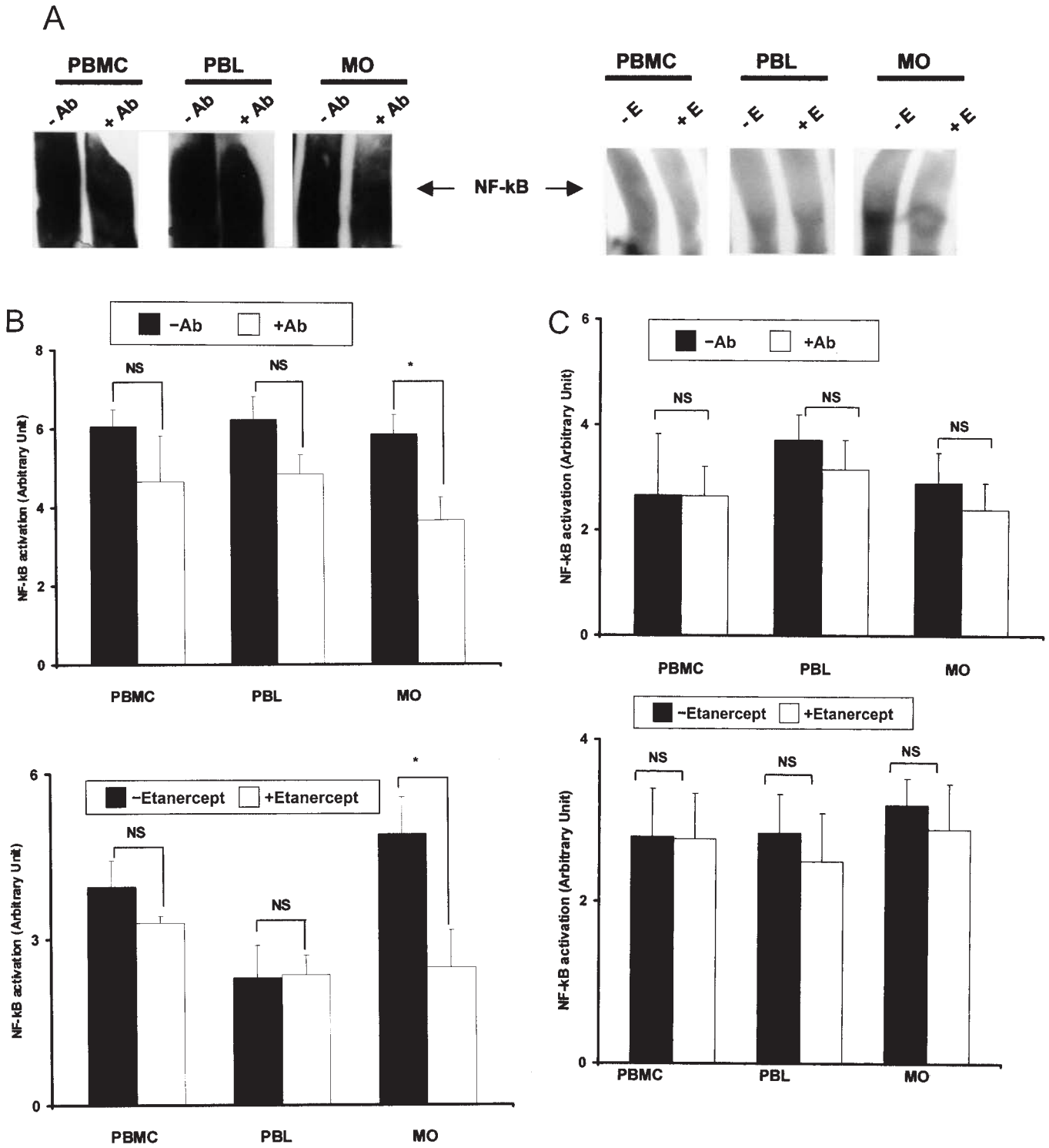

Figure 3. Increased NF-κB activation in monocytes (MO), but not in PBMC and PBL, is mediated via endogenously produced TNF-α. A. Increased NF-κB activation in monocytes of patients with RA is mediated via endogenously produced TNF-α. Nuclear extracts from primary PBMC, monocytes, and PBL isolated from the peripheral blood of patients with RA left untreated (–Ab or –E) or treated with 10 μg/ml infliximab (+Ab) or with 10 μg/ml etanercept (+E) *ex vivo* for 30 min were assayed for NF-κB activation as described in Materials and Methods. Results are representative of 5 independent experiments. B, C. Increased NF-κB activation in monocytes, but not in PBMC and PBL, of patients with RA is mediated via endogenously produced TNF-α. Histograms represent NF-κB activation measured in nuclear extracts from primary PBMC, monocytes, and PBL isolated from peripheral blood of patients with RA (B) and healthy subjects (C) left untreated (–infliximab or –etanercept) or treated with 10 μg/ml infliximab (+infliximab) or with 10 μg/ml etanercept (+etanercept) *ex vivo* for 30 min. Mean values (± SD) of independent experiments (RA n = 5; healthy subjects n = 5) are shown. *$p < 0.05$. NS: nonsignificant.

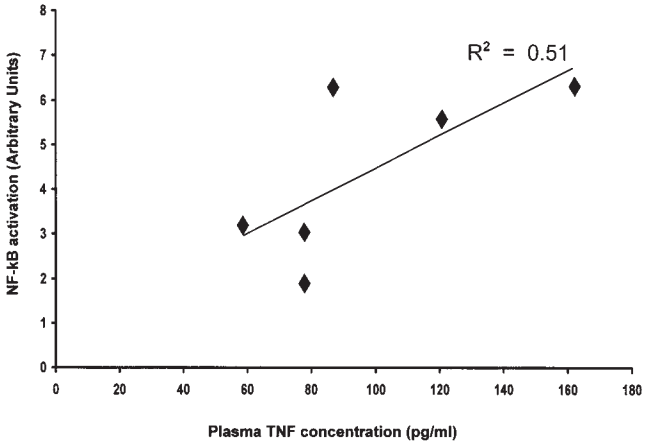

Figure 4. Correlation between plasma TNF-α levels and NF-κB activation in monocytes isolated from peripheral blood of RA patients. Plasma TNF-α levels of patients were determined by ELISA and correlated with the activation of NF-κB in the nuclear extracts of autologous monocytes.

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