Intracellular NF-κB-decrease and IKBα increase in human macrophages following CTLA4-Ig treatment

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ABSTRACT

Objective. *The transcription factor NF-κB is involved in the expression of several genes linked to the immune response, including those of pro-inflammatory cytokines. We investigated cytokine production and NF-κB expression following CTLA4-Ig (abatacept) treatment of cultured human macrophages.*

Methods. *Human THP1 cells, differentiated in macrophages, were treated with CTLA4-Ig (100, 500 μg/ml; 3,12, 24 hours). Quantitative RT-PCR analysis (qRT-PCR) of mRNA for NF-κB, IKBα and for IL-6, TNF-α, IL-1β, was performed after 3 and 12 hours from treatment. Western blot (WB) analysis for NF-κB and IKBα was performed after 24 hours from treatment.*

Results. *NF-κB gene expression was significantly downregulated (p<0.05), at 3 and 12 hours from CTLA4-Ig treatment, vs. untreated cells (cnt). IKBα resulted significantly increased vs. cnt (p<0.05), at 12 hours from CTLA4-Ig [500 μg/ml] treatment. After 3 hours, CTLA4-Ig [100 μg/ml] induced a significant decrease of TNF-α and IL-6 (p<0.05), vs. cnt and CTLA4-Ig [500 μg/ml] further reduced TNF-α (p<0.001), vs. cnt. After 12 hours from CTLA4-Ig treatment, a significant downregulation for IL-6 and IL1β expression (p<0.001), vs. cnt, was still evident. Results were confirmed by WB.*

Conclusion. *NF-κB pathway seems to be implicated in the CTLA4-Ig modulation of macrophage cytokine expression. NF-κB expression resulted downregulated while its cytoplasmatic inhibitor IKBα was increased.*

Introduction

NF-κB family members are present in the cell cytoplasm as an active heterotrimer consisting of the complex formed by NF-κB (p50 or p52)/RelA (p65) subunits and IkBα inhibitor. The phosphorylation and degradation of IkBα by proteasome, upon several membrane receptor stimulations, exposes nuclear localisation signals to the heterodimeric complex NF-κB/Rel, and allows nuclear translocation and binding to specific sequences in the DNA (1). The transcription factor NF-κB is an intracellular signalling essential for the expression of a variety of genes involved in the immune-inflammatory response, including those related to pro-inflammatory cytokines (2).

The biological agent CTLA4-Ig (abatacept), like the native CTLA-4, binds more avidly to CD80/CD86 (B7.1/B7.2 on APC) than to CD28 (T cells) molecules, and by interfering on the CD28/B7 axis represents an efficient way to dowregulate the immune/inflammatory reactivity in rheumatoid arthritis (RA) (3, 4).

Recent data showed direct effects of CTLA4-Ig on functional characteristics of human monocytes in RA patients, interfering with the migration capacity of monocytes to the synovial tissue (5). Therefore, in light of our previous studies, showing that a significant downregulation of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) was evident in cultured synovial macrophages from RA patients treated with CTLA4-Ig, we evaluated the response of the signalling involving the NF-κB complex, during the CTLA4-Ig/CD86 interaction in cultured human macrophages (6, 7).

Materials and methods

Cell culture

THP-1 human monocytes treated with phorbol myristate acetate (PMA) [0,5 μg/ml] (3 hours) were differentiated into activated macrophages as already reported (8).

The cells were seeded into culture plates ($3x10^6$ cells/well) with CTLA4-Ig [100 and 500 μg/ml] or without CTLA4-Ig (untreated cells as controls) and cultured in 5% CO_2 air humidified atmosphere at 37° for 3,12 and 24 hours.

Flow cytometric assay

CTLA4-Ig/CD86 binding was evaluated by flow cytometric assay. Macrophages were treated for 24 hours with CTLA4-Ig [100 μg/ml] or let without CTLA4-Ig (untreated macrophages as controls), then were stained with anti-human CD86 (B7.2) antibody (BD, Biosciences, NY, USA) for 30 minutes at 4°C, washed with PBS and analysed on a flow cytometer (FC500, Coulter, Hialeah, FL). Untreated macrophages were stained with IgG1 isotype anti-

body (BD, Biosciences, NY, USA), as control.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) analysis

Total mRNA was obtained with the NucleoSpin RNA/protein kit (Macherly-Nagel, Duren, Germany), by lysis of the macrophages treated with CTLA4-Ig [100, 500 μg/ml] or without CTLA4-Ig (untreated cells as controls) for 3 and 12 hours.

First-strand cDNA was synthesised using "QuantiTect Reverse Transcription Kit" (Qiagen, Milan, Italy) and quantitative real time-polymerase chain reaction (qRT-PCR) was performed using "Real MasterMix SYBER Green" detection system (Eppendorf S.r.l. Milan, Italy) on a Eppendorf Realplex 4 Mastercycler. The primers for beta-actin (housekeeping gene), NF-κBp50, NF-κBp65, IKBα, IL-6, TNF-α, IL-1β and TGFβ gene expression were supplied by PrimerDesign (PrimerDesign, UK).

The gene expression values were calculated using the comparative $\Delta\Delta C_T$ method: the values obtained correspond to a fold expression of the target gene compared to the calibrator sample (untreated cells) taken as unit value by definition (9).

Western blot (WB) analysis

Total proteins were extracted with the NucleoSpin RNA/protein kit, from macrophages treated with CTLA4-Ig [100, 500 μg/ml] or without CTLA4-Ig (untreated cells as controls) for 24 hours. The proteins, togheter with the molecular weight (Lonza, Rockland, Inc., USA), were separated by electrophoresis on a precast 12,5% tris-glycine gel, transferred into Hybond-C-nitrocellulose membrane (Life Technologies Ltd, Paisley, UK), incubated with blocking solution (PBS1x, 0.1% triton-X and 5% non-fat powdered milk) and subsequently with primary mouse antibody anti-human NF-κBp65 (diluition 1:200, Santa Cruz Biotechnology, CA, USA) and primary rabbit anti-human IKBα (1:200, Santa Cruz Biotechnology, CA, USA) at 4°C o/n. Moreover, the membranes were incubated with primary HRP conjugated antibody to human-actin (dilution 1:10000, Santa-Cruz Biotechnology, CA, USA) as controls.

Subsequently the membranes were incubated with an anti-rabbit IgG secondary antibody for IKBα (dilution 1:2000; Cell Signalling Technology, Beverly MA, USA) and an anti-mouse IgG secondary antibody for NF-κBp65 (dilution 1:5000, Cell Signalling Technology). Protein detection was performed by the enhanced chemiluminescence system (Immobilon-P, Millipore, CA, USA).

Statistical analysis

All the experiments were done in triplicate. Statistical analysis was performed by the non-parametric Wilcoxon test. $p<0.05$ was considered statistically significant.

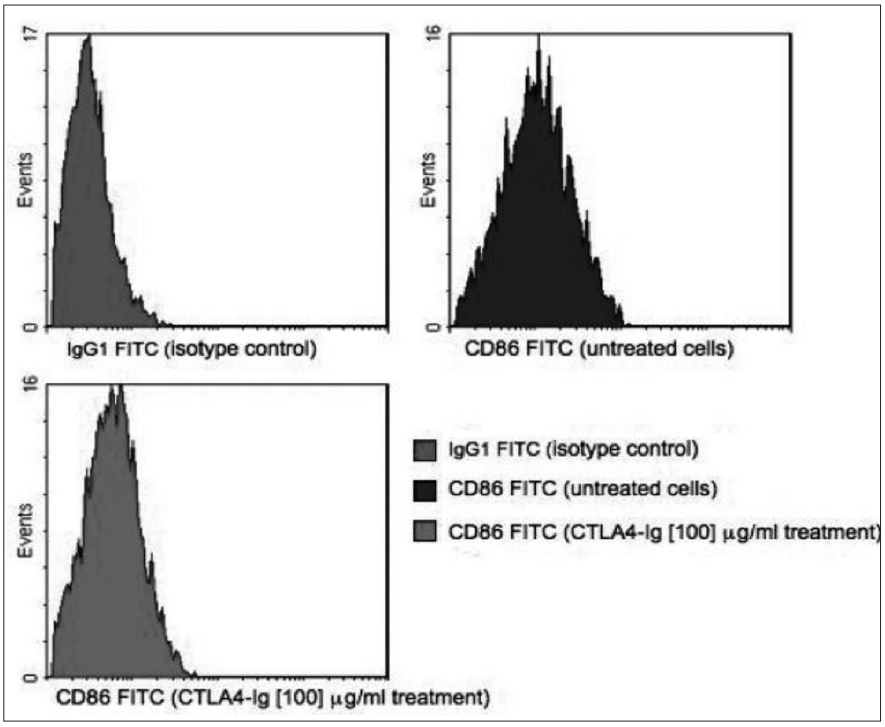

Fig. 1. CD86 flow cytometric analysis of macrophages.
A: IgG1 isotype control in untreated macrophages; **B**: CD86 staining in untreated macrophages; **C**: CD86 staining in CTLA4-Ig treated macrophages [100 μg/ml].

Results

Flow cytometric assay

Flow cytometric assay analysis confirmed a reduction of the B7.2 positivity on CTLA4-Ig -treated macrophages [100 μg/ml] when compared to CTLA4-Ig-untreated macrophages (controls), due to the truly binding of CTLA4-Ig to B7.2 with subsequent masking. Untreated macrophages, stained with IgG1 isotype, resulted negative (Fig. 1).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) analysis

The qRT-PCR analysis, at 3 and 12 hours from CTLA4-Ig [100, 500 μg/ml] treatment, showed a significant downregulation for NF-kBp50 vs. controls ($p<0.05$ for CTLA4-Ig [100 μg/ml] after 3 hours from treatment; $p<0.05$ for CTLA4-Ig [500 μg/ml] after 12 hours from treatment; $p<0.01$ for CTLA4-Ig [500 μg/ml] after 3 hours from treatment).

As expected, similar results were observed for NF-κBp65 subunit expression (data not shown): the qRT-PCR analysis, at 3 and 12 hours from CTLA4-Ig [100, 500 μg/ml] treatment showed a decrease vs. control.

Converserly, IKBα showed, an evident significant increase ($p<0.05$) after 12 hours from CTLA4-Ig [500 μg/ml] treatment, whereas less significant changes were observed after 3 hours from CTLA4-Ig treatment [100 and 500 μg/ml] vs. controls (Fig. 2 A-B).

However, qRT-PCR analysis of inflam-

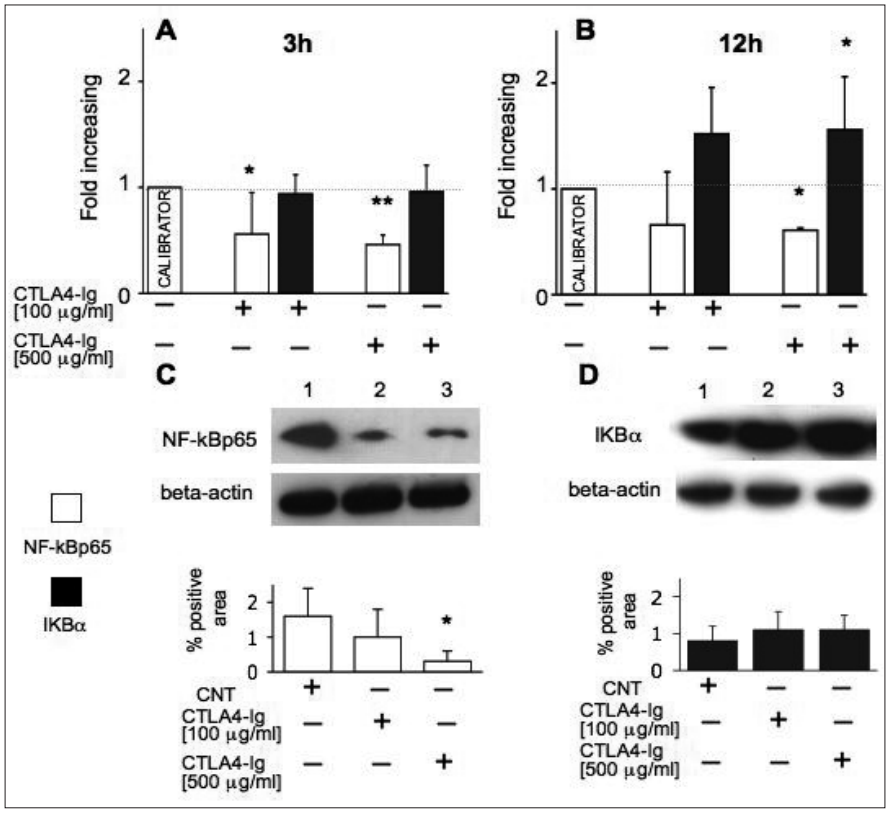

Fig. 2. qRT-PCR and WB results for NF-κB and IKBα.
A, B. Analysis by qRT-PCR of mRNA expression for NF-κB and IKBα after 3 (A) and 12 hours (B) from CTLA4-Ig treatment [100 and 500 µg/ml]. The expression values of target genes were indicated as fold expression (fold increasing) compared to those of the untreated cells (calibrator), conventionally indicated as 1. White bar is for NF-κBp50 expression, black bar is for IKBα expression.
***p<0.001 *vs*. control.
C, D. WB analysis for NF-κB (C) and for IKBα (D) in cultures of macrophages untreated (line 1:CNT), treated for 24 hours with CTLA4-Ig [100 µg/ml] (line 2) and treated for 24 hours with CTLA4-Ig [500 µg/ml] (line 3). Bar graphs represent the quantitative densitometric value of the expressed protein *vs*. actin and refer to mean values calculated from three experiments. White bar is for NF-κBp65 expression, black bar is for IKBα expression.
*p<0.05 *vs*. control.

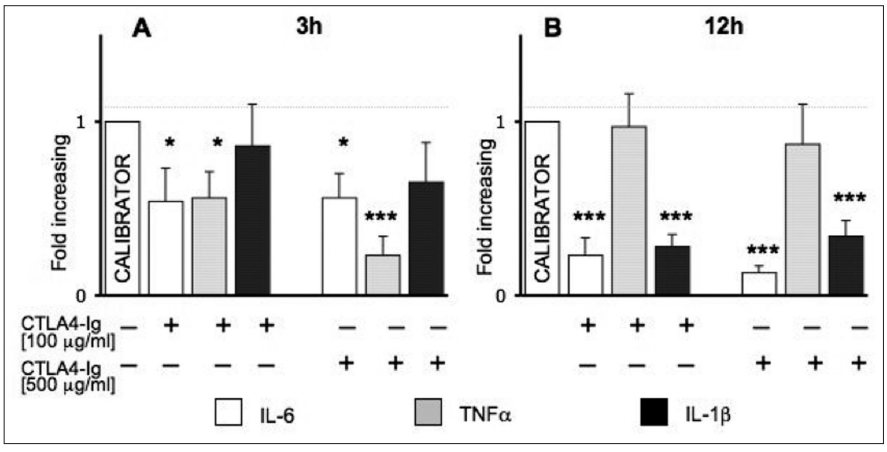

Fig. 3. qRT-PCR analysis of mRNA expression for inflammatory cytokines.
Analysis by qRT-PCR of mRNA expression for IL-6, TNF and IL-1 after 3 (A) and 12 hours (B) from CTLA4-Ig treatment [100 and 500 µg/ml]. The expression values of target genes were indicated as fold expression (fold increasing) compared to those of the untreated cells (calibrator), conventionally indicated as 1. White bar is for IL-6 expression, grey bar is for TNF-α expression and black bar is for IL-1β expression.
***p<0.001; *p<0.05 *vs*. control.

matory cytokines, after 3 hours from treatment, showed for CTLA4-Ig [100 µg/ml] a significant decrease of IL-6 and TNF-α (p<0.05) vs. controls. Interestingly, CTLA4-Ig [500 µg/ml] reduced TNF-α *vs*. controls in a larger extent (p<0.001) suggesting a dose-related response. After 12 hours from CTLA4-Ig [100, 500 µg/ml] treatment, it was still evident a significant down-regulation for IL-6 and IL-1β gene expression (p<0.001), *vs*. controls (Fig. 3). However, CTLA4-Ig [both 100 and 500 µg/ml] induced a decrease of TNFα more evident and significant after 3 hours rather then after 12 hours from treatment.

Western blot (WB) analysis
WB analysis confirmed that CTLA4-Ig treatment [500 µg/ml] was able to significantly reduce NF-κBp65 protein expression (p<0.05) and to increase IKBα expression, *vs*. cnt (Fig. 2 C-D).

Discussion

The recently described induction by CTLA4-Ig of a significant decrease for IL-6 and TNF-α expression in synovial macrophages from RA patients after 3 hours from treatment, seems now confirmed and seems mediated through to the NF-κB signalling downregulation (7, 10).
In fact, present investigation shows that, while CTLA4-Ig reduces in human macrophages the inflammatory cytokine gene expression at the same time it seems to promote after 3 hours from treatment the downregulation of the intracellular signalling linked to the NF-κB pathway, together with an increased expression of its cytoplasmatic inhibitor IKBα.
NF-κB is usually bound in the cell cytoplasm to its inhibitory protein IKBα, which prevents the nuclear translocation. The signalling pathway is activated in response to an extracellular signal, throught IKBα phosphorylation by IKB kinase (IKK), allowing the release of NF-κB. Then, NF-kB translocates into the nucleus, binds to NF-κB DNA elements and induces its target proteins, among which also IL-6, TNF-α, IL-1β as well as its own inhibitory protein, IKBα (11, 12).

Therefore, our results suggest that CTLA4-Ig, after binding to CD86 in human macrophages, it seems to induce an intracellular modulation of the NF-κB signalling pathway.

Interestingly, NF-κB and IkBα pathway expression were affected by CTLA4-Ig treatment with different kinetics when tested after 3 and 12 hours in cultured macrophages.

Although inhibition of gene expression for NF-κB was already significantly evident after 3 hours from treatment and was maintained up to 12 hours, converserly its inhibitor IkBα, started to increase after 3 hours, but was upregulated in a statistically significant manner only after 12 hours.

Recent findings have established new paradigms for innate immunoregulation, whereby for example magnesium plays a critical regulatory role in NF-κB activation and cytokine production at the level of circulating monocytes (13). In fact, MgSO(4) reduced cytokine production in intrapartum women, term and preterm neonates, demonstrating effectiveness in those at risk for inflammation-associated adverse perinatal outcomes by increasing basal IKBα levels, and upon TLR stimulation by reducing NF-κB activation and nuclear localisation.

Furthermore and recently, taurine prodrug, ethane β-sultam, was found again to reduce cellular inflammation both *in vivo* as well as *in vitro*, in primary cultures of alveolar macrophages, again through stabilisation of IKBα (14).

Therefore, CTLA4-Ig, that is close to the physiological CTLA4 molecule involved in the innate immunoregulation, seems to dowregulate the inflammatory response in activated cells by modulating the NF-κB complex.

NF-κB is one of the most ubiquitous transcription factors and functions as a central player in the chronic inflammatory disease development, mainly through IL-6 production; thus, its implication after CTLA4-Ig/B7.2 interaction becomes important in clinical applications (15).

In conclusion, several key aspects remain to be clarified before to establish a definitive correlation between the observed decreased expression of NF-κB (and increase of IKBα) and the decrease of inflammatory cytokine production, but definitely CTLA4-Ig treatment seems to interfere with the progression of both immuno-inflammatory processes.

The involvement of a more complex intracellular signalling cascade after CTLA4-Ig binding to CD86 and involving other kinases cannot be excluded at present.

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