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 (ctn). IKBα resulted significantly increased vs. ctn (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. ctn and CTLA4-Ig [500 µg/ml] further reduced TNF-α (p<0.001), vs. ctn. After 12 hours from CTLA4-Ig treatment, a significant downregulation for IL-6 and IL1β expression (p<0.001), vs. ctn, 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 IκBα inhibitor. The phosphorylation and degradation of IκBα by proteasome, upon several membrane receptor stimulations, ex- poses 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 downregulate 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 (3x10⁶ cells/well) with CTLA4-Ig [100 and 500 μg/ml] or without CTLA4-Ig (untreated cells as controls) and cultured in 5% CO₂, 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 (Mach-erly-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 ΔΔCt 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, together 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 (dilution 1:2000, Santa Cruz Biotechnology, CA, USA) and an anti-mouse IgG secondary antibody for IKBα (dilution 1:5000, Cell Signalling Technology, CA, USA) as controls.

Subsequently, the membranes were incubated with an anti-rabbit IgG secondary antibody for NF-κBp50 (dilution 1:2000, Santa Cruz Biotechnology, CA, USA) and incubated with primary HRP conjugated antibody to human-actin (dilution 1:10000, Santa Cruz Biotechnology, CA, USA) as controls. 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.

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-κBp50 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. Conversely, 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-
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Mucrotary 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 IκBα 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 IκBα.

NF-κB is usually bound in the cell cytoplasm to its inhibitory protein IκBα, which prevents the nuclear translocation. The signalling pathway is activated in response to an extracellular signal, through IκBα phosphorylation by IκB kinase (IKK), allowing the release of NF-κB. Then, NF-κB 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, IκBα (11, 12).

Fig. 2. qRT-PCR and WB results for NF-κB and IκBα.
A, B. Analysis by qRT-PCR of mRNA expression for NF-κB and IκBα 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 IκBα expression.

C, D. WB analysis for NF-κB (C) and for IκBα (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 IκBα 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.
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 IκBα 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, conversely its inhibitor IκBα, started to increase after 3 hours, but was up-regulated 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 IκBα 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 IκBα (14). Therefore, CTLA4-Ig, that is close to the physiological CTLA4 molecule involved in the innate immunoregulation, seems to downregulate 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 IκBα) 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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References