Effects of combined treatments with CTLA4-IG (abatacept), dexamethasone and methotrexate on cultured human macrophages

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Abstract

Objective

To evaluate the anti-inflammatory effect of CTLA4-Ig (abatacept) and dexamethasone (DEX) monotreatment versus their combination and adding methotrexate (MTX) on cultured human macrophages.

Methods

THP-1 cells, activated into macrophages (PMA 0.05 µg/ml), were cultured for 3 and 24 hrs with CTLA4-Ig (500 µg/ml), DEX (10^{-8} M), MTX (0.05 µg/ml), and CTLA4-Ig combined with DEX or CTLA4-Ig combined with DEX plus MTX. CTLA4-Ig/CD86 interaction was evaluated by FACS analysis. Quantitative real time-PCR (qRT-PCR), immunocytochemistry (ICC) and immunoassay (ELISA) analysis for inflammatory cytokine (IL-1β, TNF-α, IL-6) expression were performed.

Results

FACS analysis showed in macrophages treated with CTLA4-Ig alone, CTLA4-Ig-DEX and CTLA4-Ig-DEX-MTX a CD86 decrease of almost 35%, versus untreated cells (CNT). After 3 hrs, macrophages treated with DEX alone or with CTLA4-Ig-DEX or CTLA4-Ig-DEX-MTX showed a significant reduction ($p<0.05$) for all cytokines gene expression, that was still significant for IL-1β after 24 hrs ($p<0.05$). After 3 hrs, CTLA4-Ig alone significantly ($p<0.05$) reduced all cytokine genes; however, after 24 hrs still evident only for TNF-α ($p<0.05$). After 24 hrs CTLA4-Ig-DEX induced a significant decrease of gene expression ($p<0.05$) for TNF-α and IL-6, whereas CTLA4-Ig-DEX-MTX induced a decrease ($p<0.05$) limited to IL-6, versus CNT. Finally, ICC showed, after 24 hrs of CTLA4-Ig-DEX or CTLA4-Ig-DEX-MTX treatment a reduction ($p<0.05$) of IL-1β and IL-6 expression, versus CNT; DEX alone reduced only IL-1β ($p<0.05$). ELISA analysis confirmed these results.

Conclusion

CTLA4-Ig-DEX and CTLA4-Ig-DEX-MTX combined treatments, decreased at any level the inflammatory cytokine expression more efficiently then monotreatments on activated cultured human macrophages.

Key words

CTLA4-Ig, dexamethasone, methotrexate, macrophages, rheumatoid arthritis, glucocorticoids

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Background

To date there are numerous effective therapeutic agents available for treating rheumatoid arthritis (RA) and chronic inflammation, such as biologic agents that may exert disease-modifying effects (DMARD) (1).

Among biological agents, CTLA4-Ig (abatacept) is constructed by genetically fusing the external domain of human CTLA4 and a modified fragment of the Fc domain of human immunoglobulin G1 (IgG1) and, like the native CTLA4, binds to CD80/CD86 molecules blocking this costimulatory pathway in several cell populations (2). In clinical trials, abatacept was found effective in treating the signs and symptoms of RA, as well as in reducing joint structural damage (3-5).

Glucocorticoids (GCs) may exert disease-modifying effects even at low doses, even if their use in RA it is not suggested as full doses monotherapy, owing to their adverse event profile (6). Glucocorticoids are used in RA treatment, mainly with the intention to bridge the time period between the start of a newly initiated or changed DMARD regimen and the time point at which this combination will become clinically effective (bridging therapy) (7). As matter of fact, low-dose GC treatment in association with standard DMARDs is effective in RA in inducing high remission rates, earlier and more persistently then monotherapies (8, 9).

Among conventional DMARDs, low dose methotrexate (MTX) is one of the most common immunosuppressants used in RA and other inflammatory conditions (10, 11). However, in early RA, MTX was found efficacious in clinical trials, provided considerably greater long-term effectiveness with considerably greater safety in combined treatment with GCs (12). In addition, MTX in combination with abatacept resulted clinically effective for patients with RA, which were refractory to MTX alone (13, 14).

Of note, from the earliest studies, abatacept in combination with synthetic DMARDs and low dose GC (over 70% of treated patients in almost all studies) was well tolerated and improved physical function and physician- and patient-reported disease outcomes (15).

Besides its effect on T cells, CTLA4-Ig has been demonstrated to induce reverse signalling on human RA synovial macrophages upon binding to CD80/CD86, masking its expression and dowregulating the production of proinflammatory cytokines (16-18).

In addition, previous published results suggest that the combination therapy between DMARDs and GCs seems to play a synergistic effect in the inflammatory cytokine gene modulation in RA patients (19).

To test possible *in vitro* effects of combination treatments, present study investigated for the first time the effects of CTLA4-Ig and dexamethasone (DEX) monotreatment *versus* their mutual combination and *versus* their combination with MTX, on immune-inflammatory cytokine production by cultured human activated macrophages.

Methods

Cell culture

THP-1 human monocytes were differentiated into activated macrophages with 0.05 μg/ml phorbol myristate acetate (PMA, Sigma, Milan, Italy) treatment for 24 hours, as already reported (14).

Therefore, the cells were seeded into culture plates ($3x10^6$ cells/well) or into flexiperm chamber slides ($3x10^5$ cells/well) and cultured in 5% CO_2 air humidified atmosphere at 37°C for 3 or 24 hours with different conditions.

Activated macrophages were treated with CTLA4-Ig alone (500 μg/ml) as previously described, with the GC dexamethasone alone (DEX, 10^{-8} M) or with MTX alone (0.05 μg/ml); some aliquots were untreated and used as controls (20).

Combination treatments included CTLA4-Ig (500 μg/ml) combined with DEX (10^{-8} M) and CTLA4-Ig (500 μg/ml) combined with DEX (10^{-8} M) plus MTX (0.05 μg/ml).

Flow cytometric assay

CTLA4-Ig/CD86 interaction was evaluated by flow cytometric assay. Activated macrophage cultures treated as previously described for 24 hours, were harvested and stained with anti-human CD86 (B7.2) mouse antibody

Table I. Quantitative FACS analysis for CD86 protein expression on human activated macrophages untreated (CNT) and treated for 24 hours with: dexamethasone, DEX (10^{-8} M) alone; CTLA4-Ig (500 μg/ml) alone; methotrexate, MTX (0.05 μg/ml) alone; CTLA4-Ig-DEX combination (CTLA4-Ig + DEX) and CTLA4-Ig-DEX-MTX combination.

	CD86 Fluorescence	
	% gated	X-mean
CNT (untreated cells)	47.0	9.8
DEX (10^{-8}M)	24.0	7.7
CTLA4-Ig (500 μg/ml)	33.0	7.5
MTX (0.05 μg/ml)	49.0	9.0
CTLA4-Ig + DEX	29.0	8.2
CTLA4-Ig + DEX + MTX	28.6	7.4

(BD, Biosciences, NY, USA) for 30 minutes at 4°C. Then, the cells were washed with phosphate buffered saline (PBS1x) and finally analysed at fluorescence-activated cell sorting (FACS) (FC500, Coulter, Hialeah, FL).
Background fluorescence will be assessed on untreated activated macrophages using appropriate isotype- and fluorochrome-matched control antibody (IgG1 isotype control, BD, Biosciences, NY, USA). The fluorescence was measured by considering two main parameters: the percentage of positive cells (%) and the mean fluorescence intensity (MFI).

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 all the cultured activated macrophages, treated as previously described for 3 and 24 hours.
First-strand cDNA was synthesised using "QuantiTect Reverse Transcription Kit" (Qiagen, Milan, Italy) and subsequent 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), IL-1β, TNF-α, and IL-6 gene expression were supplied by Primer Design (PrimerDesign, UK).
The gene expression values were calculated using the comparative $\Delta\Delta C_T$ method: the values obtained correspond to a fold expression (fold increasing) of the target gene compared to the calibrator sample (untreated cells) taken as unit value by definition (21).

Immunocytochemistry (ICC) assay
After removing the flexichambers, the slides with activated macrophages, treated as previously described for 24 hours, were incubated in a 3% H_2O_2 solution for 15 minutes to block endogenous peroxidases. Then the slides were incubated with anti-cytokine antibodies (anti-IL1β, TNF-α, anti-IL6, diluition 1:100; Santa Cruz Biotechnology, CA, USA) at room temperature for 45 minutes. Linked antibodies were detected by a biotinylated universal secondary antibody and subsequently by a horseradish peroxidase streptavidin complex (Vector Laboratories, Burlingame, CA, USA). Each step was followed by two washes in PBS1x. The staining reaction was developed by the diaminobenzidine system (DAB, DakoCytomation, Carpinteria, CA, USA) and the cells were also stained with haematoxylin. Finally, image analysis was performed using the Leica Q-Win Image Analysis System (Leica, Cambridge, UK).

Immunoassay multiplex system analysis
Culture surnatants were harvested from activated macrophages, treated as previously described for 24 hours, and were immediately stored at -80°C.
Subsequently, TNF-α, IL-6, IL-10 and γIFN cytokine levels were evaluated in culture surnatants, by a immunoassay multiplex system, formatted on magnetic beads (Bio-Plex Pro Cytokine factor assay, Bio-Rad Laboratories, Inc., Milan, Italy), following the manufacturer's instructions.

Statistical analysis
The statistical analysis was performed using the non-parametric Wilcoxon matched pair test (p-values <0.05 were considered significant). All the experiments were done at least in triplicate.

Results

CD86 protein expression and CTLA4-Ig/CD86 interaction on activated macrophages
Quantitative FACS analysis revealed 47% of CD86-positivity on untreated macrophages (CNT), whereas CD86 expression on cells treated with CTLA4-Ig (500 μg/ml) alone showed a decrease of 30%, compared to the CNT. These results suggest a CTLA4-Ig/CD86 interaction and related fluorescence decrease due to molecule masking on cell surface (16). The CD86 expression on cells treated with DEX (10^{-8} M) alone showed a decrease of 49%, compared to the CNT.
CTLA4-Ig (500 μg/ml) combined with DEX (10^{-8} M) treatment, and CTLA4-Ig (500 μg/ml) combined with DEX (10^{-8} M) plus MTX (0.05 μg/ml) treatment induced in the activated macrophages a CD86 decrease of 38% for both combinations, compared to the CNT.
Interestingly, MTX alone did not induce changes in membrane protein expression of CD86. Finally, not relevant changes in MFI were observed for all conditions evaluated (Table I). A negative result was obtained for the specific isotype control analysed.

QuantitativeRT-PCR cytokine gene expression
Interestingly, after 3 hours, all treatments induced on activated macrophages a significant decrease (p<0.05) of all assayed cytokines, compared to the untreated cells (CNT) (Fig. 1A).
However, after 24 hours, DEX (10^{-8} M) alone and CTLA4-Ig/DEX and CTLA4-Ig/DEX plus MTX combined treatments still showed the most significant reduction in gene cytokine expression only for IL-1β (p<0.05). Otherwise, after 24 hours of treatments, gene expression for TNF-α and IL-6 was not inhibited by DEX (10^{-8} M) alone treatment, whilst still significantly decreased (p<0.05) after CTLA4-Ig/

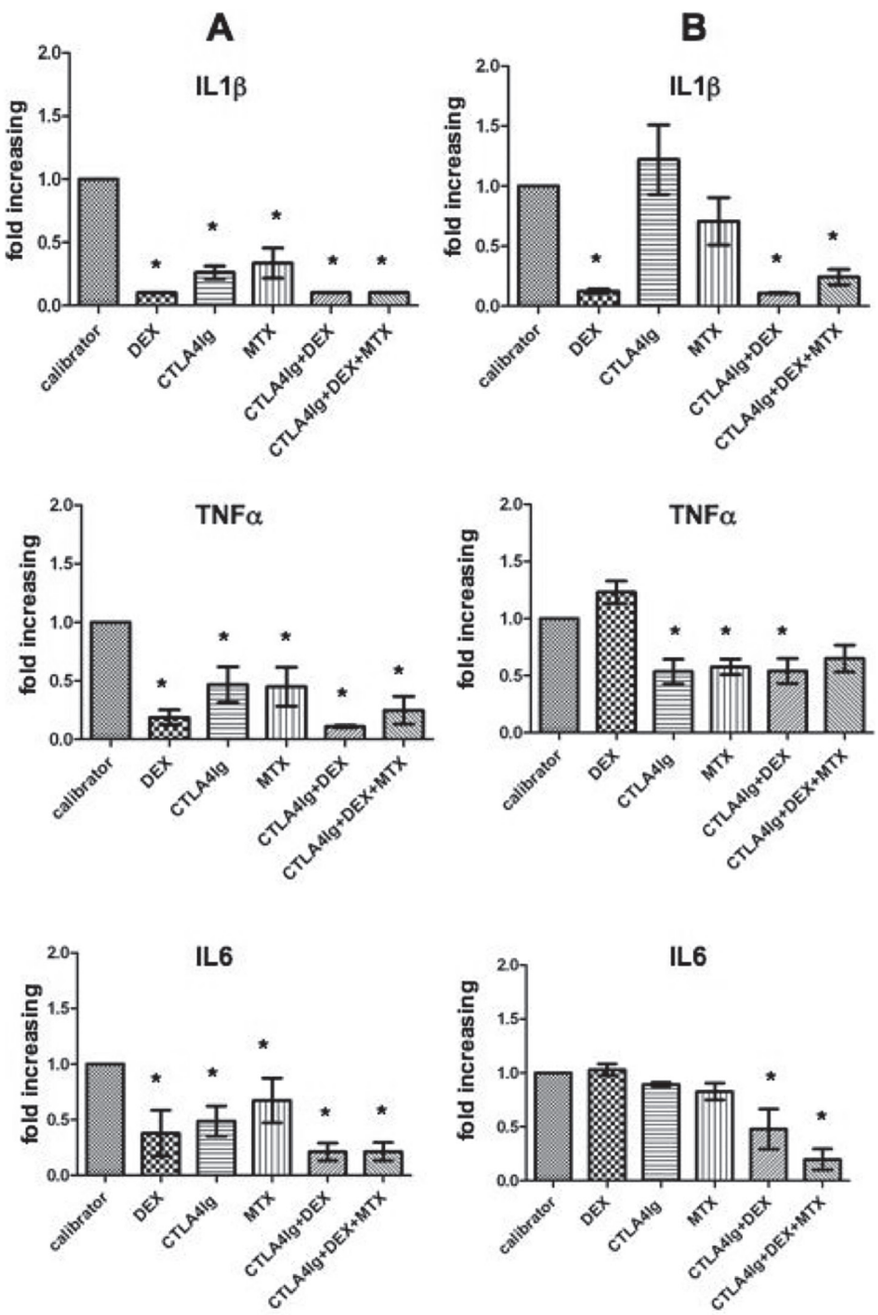

Fig. 1. Quantitative RT-PCR analysis for IL-1β, TNF-α and IL-6 gene expression on human activated macrophages treated for 3 hours (**A**) and 24 hours (**B**) with: dexamethasone, DEX (10^{-8} M) alone; CTLA4-Ig (500 μg/ml) alone; methotrexate, MTX (0.05 μg/ml) alone; CTLA4-Ig-DEX combination and CTLA4-Ig-DEX-MTX combination. 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, CNT) taken as unit value by definition.
The statistical analysis was performed using the non-parametric Wilcoxon matched pair test. All the experiments were done at least in triplicate. *p<0.05; **p<0.01.

DEX treatment. Similarly, CTLA4-Ig/DEX plus MTX combined treatment induced, after 24 hours, a decrease of TNF-α (not significant) and of IL-6 (p<0.05) gene expression (Fig. 1B).
In addition, after 24 hours, CTLA4-Ig (500 μg/ml) alone and MTX (0.05 μg/ml) alone treatments still induced a significant reduction (p<0.05) limited to TNF-α, compared to CNT (Fig. 1B).

ICC cytokine protein expression
After 24 hours, CTLA4-Ig/DEX and CTLA4-Ig/DEX plus MTX combined treatments showed a significant reduction of IL-1β and IL-6 protein expression (p<0.05). Whereas, DEX (10^{-8} M) alone still significantly reduced only IL-1β (p<0.05) (Fig. 2. A-B). Therefore, after 24 hours, TNF-α protein expression resulted not significant reduced by all the treatments (Fig. 2. A-B).
Interestingly, after 48 hours CTLA4-Ig/DEX treatment still reduced IL-1β (p<0.05) and TNF-α production (Fig. 3).

Immunoassay multiplex system cytokine analysis
After 24 hours of DEX (10^{-8} M) alone and CTLA4-Ig/DEX and CTLA4-Ig/DEX plus MTX combined treatments, immunoassay multiplex system analysis showed that TNF-α levels in culture surnatants from activated macrophages decreased, compared to the untreated cells (CNT). Moreover, IL-6 levels in culture surnatants from activated macrophages, decreased only after 24 hours of CTLA4-Ig/DEX plus MTX combined treatment, compared to the controls, whilst resulted unchanged with all the other tested conditions. Finally, we observed that IL-10 and γIFN levels resulted unchanged, after all performed treatments (Fig. 4).

Discussion

Both CTLA4-Ig/DEX and CTLA4-Ig/DEX plus MTX combined treatments, induced a reduction in CD86 expression and a significant anti-inflammatory effect on human activated macrophages, by decreasing proinflammatory cytokine production.
The results seem due to both the CTLA4-Ig/CD86 blocking effects on cell costimulation, and the genomic downregulatory effects exerted by GCs on cytokine gene expression (19).
Our previous studies already reported the rapid interaction of CTLA4-Ig single treatment with cultured synovial macrophages from RA patients, as well as downregulatory effects on cytokine production (16-18). Synovial macrophages treated with CTLA4-Ig alone showed a rapid downregulation of IL-1β, TNF-α, and IL-6 inflammatory cytokine gene expression and production, already evident after 3 hours from treatment (16). Similarly, we demonstrated that, also on human activated macrophage cultures, CTLA4-Ig single

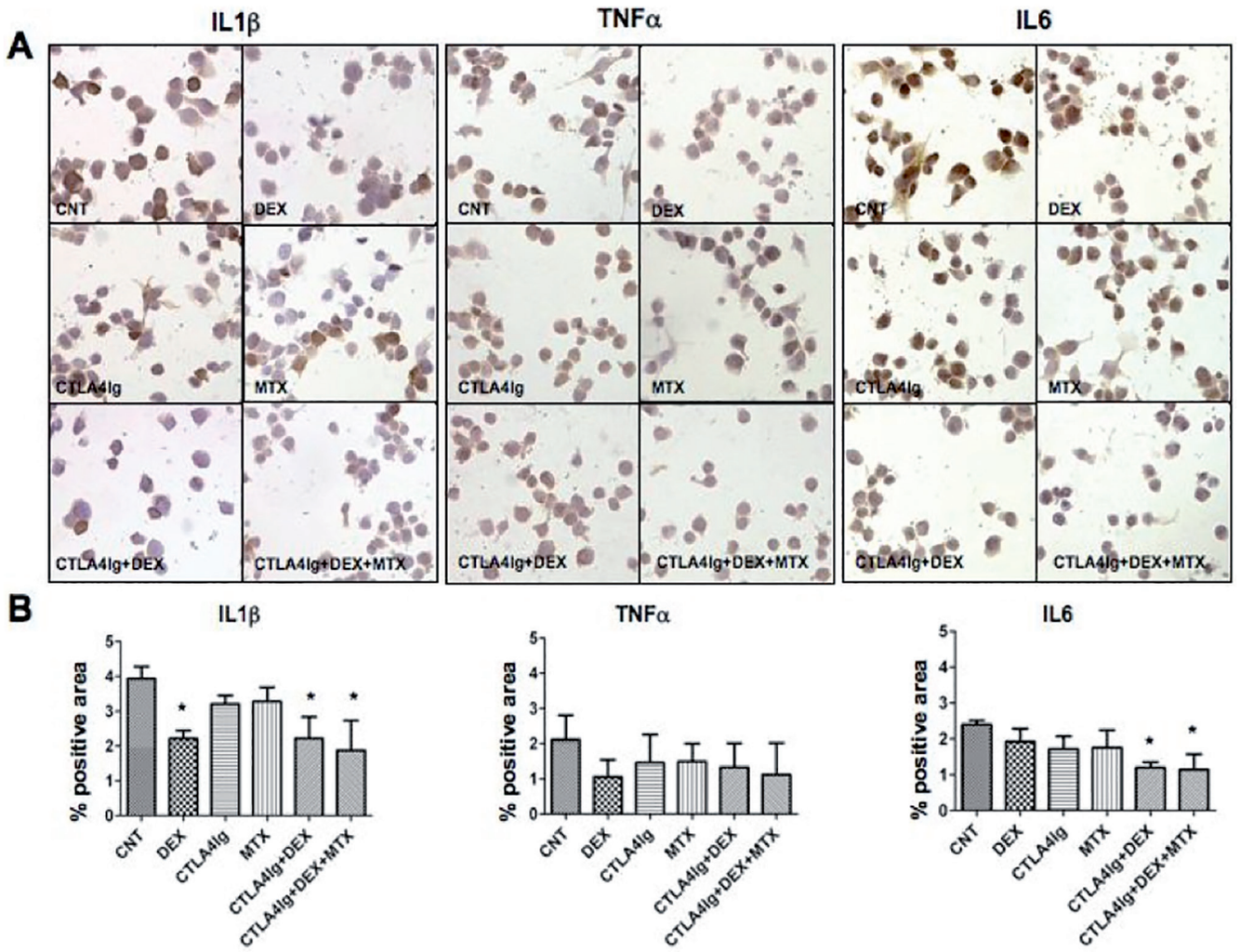

Fig. 2. **A**. ICC images for IL-1β, TNF-α and IL-6 expression on human macrophages untreated (CNT) and treated for 24 hours with: dexamethasone, DEX (10^{-8} M) alone; CTLA4-Ig (500 μg/ml) alone; methotrexate, MTX (0.05 μg/ml) alone; CTLA4-Ig-DEX combination and CTLA4-Ig-DEX-MTX combination. **B**. Relative ICC image analysis for IL-1β, TNF-α and IL-6 on human macrophages untreated (CNT) and treated for 24 hours with: dexamethasone, DEX (10^{-8} M) alone; CTLA4-Ig (500 μg/ml) alone; methotrexate, MTX (0.05 μg/ml) alone; CTLA4-Ig-DEX combination and CTLA4-Ig-DEX-MTX combination. Leica Q-Win Image Analysis System, Leica, Cambridge, UK. Statistical analysis was performed using the non-parametric Wilcoxon matched pair test. All the experiments were done at least in triplicate. *p<0.05.

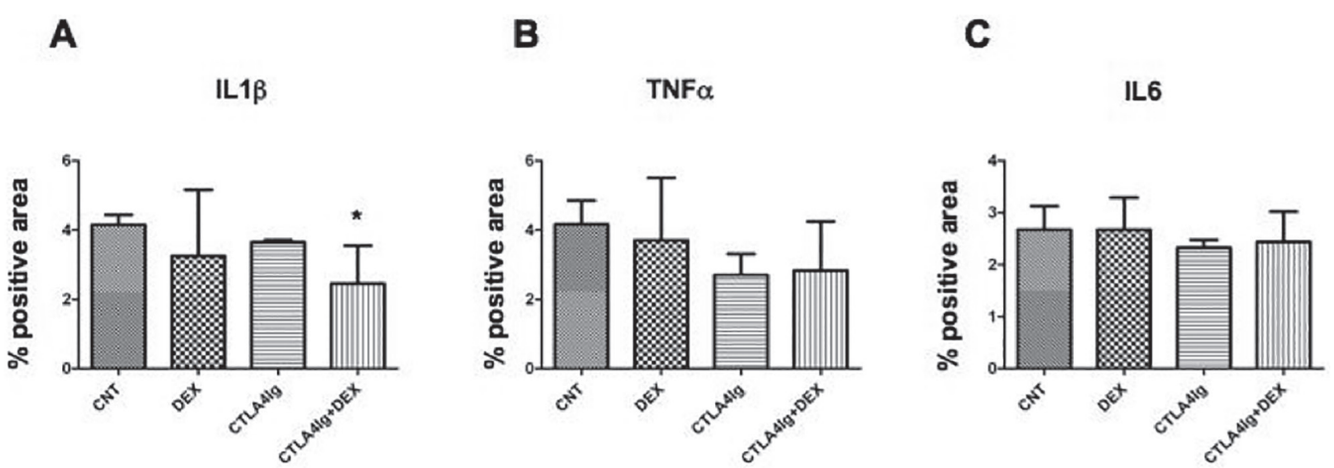

Fig. 3. ICC image analysis for IL-1β (**A**), TNF-α (**B**) and IL-6 (**C**) expression on human macrophages untreated (CNT) and treated for 48 hours with: dexamethasone, DEX (10^{-7} M) alone; CTLA4-Ig (500 μg/ml) alone; CTLA4-Ig-DEX combination. Leica Q-Win Image Analysis System, Leica, Cambridge, UK. Statistical analysis was performed using the non-parametric Wilcoxon matched pair test. All the experiments were done at least in triplicate. *p<0.05.

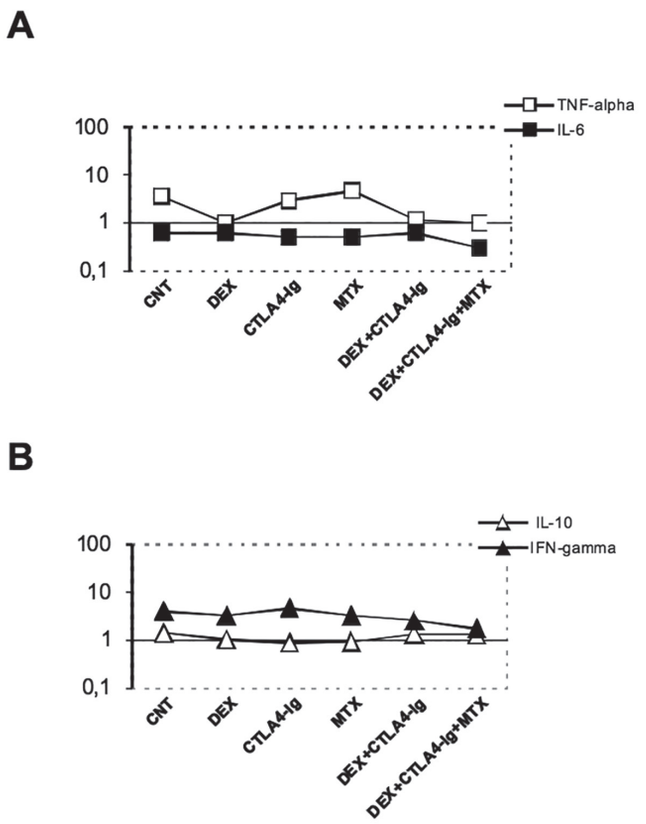

Fig. 4. Immunoassay multiplex system analysis of TNF-α, IL-6, IL-10 and γIFN cytokine levels in culture surnatants from human macrophages untreated (CNT) and treated for 24 hours with: dexamethasone, DEX (10^{-8} M) alone; CTLA4-Ig (500 μg/ml) alone; methotrexate, MTX (0.05 μg/ml) alone; CTLA4-Ig-DEX combination and CTLA4-Ig-DEX-MTX combination. Results were expressed in pg/ml.

treatment already after 3 hours from treatment, induced a reduction in these cytokine production.

The fusion protein CTLA4-Ig has high affinity for the costimulatory molecule CD86, expressed on cell surface of activated macrophages, and this *in vitro* interaction on activated macrophages, mask the epitope that could not interact with the antiCD86-FITC antibody used during the flow cytometric analysis. Therefore, the interaction between CTLA4-Ig and CD86 resulted inversely correlated to the measure of the fluorescence, as consequence a decrease of fluorescence for CD86 by cells treated with CTLA4-Ig should reflect a CTLA4-Ig/CD86 interaction.

In addition, immunocytochemistry experiments for IL-6 and TNF-α expression on cultured macrophages showed no downregulation after 24 hours of CTLA4-Ig treatment, in the testing condition of pretreatment with anti-CD86 blocking antibodies, confirming that the effects on synovial macrophages were really due to the CD86/CTLA4-Ig binding (16).

We previously observed the same results after short CTLA4-Ig treatments (45 minutes) also on activated macrophages by immunofluorescence analysis (18).

The present study confirmed the significant inhibitory effect of DEX, even at low dose (10^{-8}M), on the expression of CD86 on APCs (22).

Moverover, already after 3 hrs, a fast downregulation of gene expression for all proinflammatory cytokines evaluated was observed for DEX alone or combined treatments. Previous data demonstrated that GCs downregulate gene expression of immune-inflammatory molecules in RA patient serum (19).

GCs can rapidly reduce RA disease activity and may hence contribute to achieving a better outcome, since they are effective as bridging/synergistic therapy and should be always included for the use of non-biologic and biologic DMARDs in the RA treatment (7, 8).

Interestingly, in RA patients inadequate responders to MTX, the combination of long term abatacept with low dose GC, was found to induce inhibition of structural damage progression even over 5 years (23, 24).

Antiproliferative agents, such as MTX, can hinder DNA synthesis by blocking activity of essential enzymes implicated in purine and pyrimidine synthesis, however in present study MTX alone did not induce changes in membrane protein expression of CD86.

MTX is recognised as an anchor drug in RA and MTX and DMARDs offer an avenue for improving RA treatment; MTX is characterised by proven efficacy in combination DMARD strategies, but relatively slow onset of action (weeks) (25). Clinical efficacy of CTLA4-Ig significantly increased even after 24 weeks in patients with concomitant MTX use (26).

In real-world practice, however, approximately one-third of RA patients receiving biologics are on monotherapy mainly because of intolerance of, or noncompliance with, MTX.

Therefore, the results of the present *in vitro* study, might support the improved clinical conditions observed in RA patients treated with combined CTLA4-Ig and GCs, and should add important informations to the current use of combination therapies in RA treatment, following the EULAR recommendations (27).

In particular, the successfull combination of CTLA4-Ig with GCs seems today one of the most promising therapeutical approaches to early RA, by considering possible rapid and additive effects exerted by both agents (28, 29).

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