Clinical and Experimental Rheumatology 2011; 29: 697-700.

Anti-IL-6 receptor antibody (tocilizumab): a B cell targeting therapy

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Received on December 8, 2010; accepted in revised form on March 8, 2011.

Key words: tocilizumab, B cells, IL-6, rheumatoid arthritis, TGF-β

Competing interests: none declared.

ABSTRACT

Background. *IL-6 mediated inflammation is induced by binding to IL-6 receptor (IL-6R) or IL-6/IL-6R complex binding gp130. Tocilizumab, a recombinant humanised monoclonal antibody that acts as IL-6R antagonist has been recently introduced for the treatment of rheumatoid arthritis (RA).*

Objective. *To evaluate whether tocilizumab therapy may induce B cells to undergo phenotypic changes compatible with regulatory function.*

Patients and methods. *B cells from treated RA patients were isolated before and after 3 months of treatment with tocilizumab and were stained for the expression of intracellular TGF-β, IL-10, membrane CD69, and MHC-II. These markers were assessed in $CD25^{high}$ B cells considered to belong to a regulatory/suppressive subset of B cells. All markers were expressed in mean flow cytometry intensity (MFI), with results given in mean ± SEM. Data was compared before and after tocilizumab treatment.*

Results. *Clinical improvement was noted three months following the initiation of tocilizumab, namely: DAS improvement from 6.8±0.3 at baseline to 3.1±0.4, $p<0.002$, and ESR decrease from 44.4±8.6 at baseline to 7.4±2.3, $p<0.006$. This clinical benefit was found to occur in association with the expansion of a B cell subset with regulatory properties namely: the expression of intracellular TGF-β in $CD25^{high}$ B cells was significantly increased (from 5.2±2.3 at baseline to 8.1±2.8; $p<0.02$); In addition, the expression of MHC-II and of CD69 on B cells were significantly reduced (from 9.1±2.2 at baseline to 4.2±0.4; $p<0.04$), and (from 7.6±2.4 at baseline to 2.7±0.7; $p<0.03$) respectively.*

Conclusion. *The present finding of a shift in B cell properties following tocilizumab treatment, namely the increase in TGF-β expression and the alteration in the activation status (CD69 expression) and APC properties (MHC-II expression) in $CD25^{high}$ B cells, suggests that the induction/expansion of B regulatory cells may be one of the mechanisms by which tocilizumab may possibly produce its beneficial clinical effects.*

Introduction

Interleukin-6 (IL-6) is a frontier cytokine in immune regulation response, generation of acute-phase reactions and induction of inflammation. Overproduction of IL-6 is involved in many inflammatory autoimmune diseases such as rheumatoid arthritis (RA), systemic juvenile idiopathic arthritis (sJIA) and systemic lupus erythematosus (SLE) (1, 2). Levels of serum IL-6 were reported to be increased in association with SLE disease activity (lupus nephritis, serositis), and the production of relevant autoantibodies (anti-dsDNA) (3). Further, increased IL-6 in SLE serum was shown to induce the differentiation and maturation of dendritic cells, affecting thereby T cell differentiation and activation (4). Increased levels of serum IL-6 have also been reported to shift the balance between IL-17-producing Th17 cells and T regulatory cells, thus contributing to the development and disease activity of RA (5).

IL-6 mediated inflammation is induced by binding to IL-6 receptor (IL-6R) or IL-6/IL-6R complex binding to gp130, and/or via intracytoplasmic signalling through gp130 (6).

In consideration of the above, interruption of the IL-6/IL-6R loop has become a reasonable therapeutic strategy for many IL-6 mediated diseases. Tocilizumab, a recombinant humanised monoclonal antibody that acts as an IL-6R antagonist thus inhibiting IL-6 activity, is currently used for treating RA, sJIA, and SLE (7). As monotherapy and in combination with methotrexate (MTX), it has been shown to be effective in patients who have failed MTX alone or other disease-modifying antirheumatic drugs (8).

Apart from their role as producers of autoantibodies, B cells are appreciated as antigen presenting cells and activators of T effector cells. When efficiently stimulated by sequential B cell receptor and CD40 stimulation, B cells proliferate and secrete other pro-inflammatory cytokines, *e.g.* IL-6, which can act as autocrine growth and differentiation factors and also serve to amplify ongoing inflammatory responses. On the other hand, B cells also function as regulatory cells by produc-

ing inhibitory cytokines such as TGF-β and/or IL-10. In a previous study, human $CD25^{high}$ B cells were shown to secrete higher levels of IL-10 *vs*. $CD25^{low}$ B cells, suggesting this subset of cells to be of both memory and of immune-regulatory properties (9). B regulatory cells have been identified in many animal and human models and shown to down-regulate Th1 and dendritic cells, thus contributing to the maintenance of self-tolerance.

Following treatment with tocilizumab, one might expect to achieve a reduction in both B cell activity and antigen presenting properties, possibly mediated through the interruption of the autocrine IL-6/IL-6R loop on B cells. In the present study, we demonstrate for the first time that tocilizumab therapy induces B cells to undergo phenotypic changes compatible with regulatory function, which may partially explain the clinical response.

Patients and methods

Freshly purified B lymphocytes were isolated from 10 active RA patients with inadequate clinical response to methotrexate, at baseline and 3 months following add on tocilizumab. All the patients had been on a stable dose of methotrexate of 15 mg/week for at least 3 months prior to the trial, which they continued; all other disease modifying drugs were discontinued 2 months previously; all were on a stable dose of corticosteroids as prednisone ≤10 mg/day before and throughout the study. At initiation of tocilizumab therapy all of the patients were in clinical flare, with more than 10 swollen and 10 tender joints and erythrocyte sedimentation rate (ESR) ≥35 mm/hour (ESR was preferred as tocilizumab rapidly reduces C-reactive protein level even in the absence of clinical response.) Patients' clinical status was monitored by the standardised disease activity (DAS) 28 score (10). IgM rheumatoid factor (IgM-RF) and anti-CCP antibodies were assessed (commercial assays) in all RA patients.

Peripheral blood mononuclear cells (PBMCs) were isolated from study participants' on a Lymphoprep density gradient (Axis-Shield, Oslo, Norway). Purified B lymphocyte fraction was collected by positive selection using CD22 MicroBeads and magnetic cell separation kit (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions, achieving >95% purity.

Informed consent was obtained, and the study was approved by the local Helsinki committee at Bnai Zion Medical Center, Haifa, Israel.

Flow Cytometry

Freshly purified B lymphocytes were stained for extracellular markers with anti-human CD19, CD25 and CD69 or MHCII Abs (Immunotech, Beckman coulter co. Marseille, France). For detection of intracellular TGF-β or IL-10, cells were fixed and permeablised with cell permeabilisation kit (Caltag Lab, An Der Grub, Austria) then stained with anti-TGF-β (IQ products, Groningen, Netherlands) or anti-IL-10 (Biolegend, San Diego, CA, USA). As a control, B cells were stained with isotype - matched control Abs. These markers were assessed in $CD25^{high}$ B cells considered to belong to a regulatory/suppressive subset of B cells.

Data was acquired using Flow Cytometry (FC 500, Beckman coulter co. Marseille, France). All markers were expressed in mean flow cytometry intensity (MFI), with results given in mean ± SEM.

Statistical analyses

Expression of MHCII, CD69 and TGF-β on $CD25^{high}$ B cells population was compared between the two blood samples of each patient, and analysed by Wilcoxon signed rank test. *P*-values of 0.05 or less were considered to be statistically significant. Data are expressed as mean values of MFI ± SEM.

Results

Clinical benefit was noted in all ten patients three months following the initiation of tocilizumab, namely DAS improvement from 6.8±0.3 at baseline to 3.1±0.4, $p<0.002$ (Fig. 1A). All patients achieved a clinical response comparable to an ACR 50 response. ESR decreased from 44.4±8.6 at baseline to 7.4±2.3, $p<0.006$ (Fig. 1B). Positive IgM-RF was found in 6 of 10 patients and anti-CCP antibodies in 8 of 10 patients. However, tocilizumab was found to be equal clinically beneficial in both negative and positive RF patients.

Clinical improvement was found to occur in association with the alteration in B cell activity and APC properties and the shift and/or expansion of the B cell subset with regulatory properties namely: the expression of intracellular TGF-β in $CD25^{high}$ B cells was significantly increased (from 5.2±2.3 at baseline to 8.1±2.8; $p<0.02$) (Fig. 1C); the expression of MHC-II on B cells was significantly reduced (from 9.1±2.2 at baseline to 4.2±0.4; $p<0.04$) (Fig. 1D). In addition, the expression of CD69 also decreased (from 7.6±2.4 at baseline to 2.7±0.7; $p<0.03$). The expression of intracellular IL-10 in primary (non-stimulated) B cells was too low for comparison.

Discussion

Our unique finding of a shift in B cell properties following tocilizumab treatment, namely the increase in TGF-β expression and the alteration in their activation status (CD69 expression) and APC properties (MHC-II expression) in $CD25^{high}$ B cells, suggests that the induction/expansion of B regulatory cells may be one of the mechanisms by which tocilizumab may possibly produce its beneficial clinical effects. The precise mechanism by which a dissociation of IL-6/IL-6R complex and/or the neutralisation of IL-6 signaling could possibly lead to the induction or expansion of B cells with regulatory characteristics requires better understanding. Further studies are required to establish this important preliminary data and to list tocilizumab among other therapies: a B cell targeting drug.

Tocilizumab apparently induces its beneficial clinical effect by many well established mechanisms, namely by binding to both soluble IL-6 receptor (sIL-6R) and membrane bound IL-6 (mIL-6) as well as inhibiting IL-6 binding to its receptor. Tocilizumab has been shown to dissociate IL-6 and sIL-6R from their preformed complex and suppress the IL-6/IL-6R complex-induced proliferation of human gp130

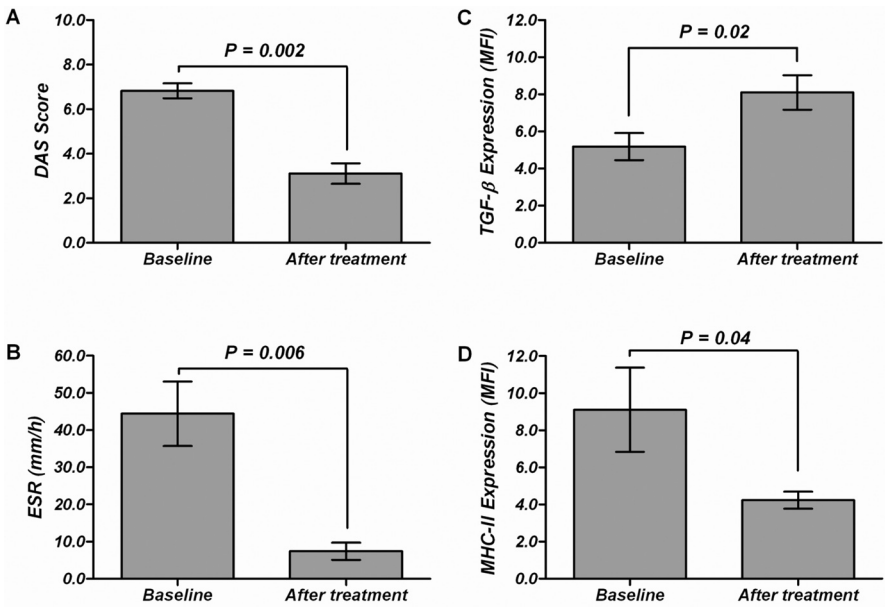

Fig. 1. - **A**. DAS was significantly reduced 3 months after tocilizumab treatment (6.8±0.3 at baseline to 3.1±0.4; $p<0.002$). **B**. ESR level was significantly decreased in association with clinical improvement following tocilizumab treatment (44.4±8.6 at baseline to 7.4±2.3; $p<0.006$). **C**. TGF-beta expression in $CD25^{high}$ B cells was significantly increased (5.1±0.7 at baseline to 8.1±0.9; $p<0.02$) 3 months after tocilizumab treatment. **D**. MHC II expression on $CD25^{high}$ B cells was significantly decreased (9.1±2.2 at baseline to 4.2±0.45; $p<0.04$) 3 months after tocilizumab treatment.

transfected cells (11). As an autocrine survival, growth and proliferative factor for B cells, IL-6 can up-regulate many important receptors such as glucocorticoid receptor, thereby increasing B cell pro-inflammatory activity (12). The environmental factors or cytokines involved in the induction of regulatory B cells are yet to be well identified.

Trying to identify additional regulatory mechanisms, the number of IL-10-producing B cells was found to be significantly increased when B cells were co-cultured with B cell-activating factor (BAFF). In that study, BAFF-induced IL-10-producing B cells, which are $CD1d^{high}$ and $CD86^{high}$, possessed a regulatory function both *in vitro* and *in vivo*. Namely, they inhibited Th1 proliferation and cytokine secretion (13). This is in keeping with our previous finding of the change in macrophage function after rituximab treatment in patients with RA. In association with clinical improvement, the expression of BAFF, IL-10 and CD86 mRNA in human monocyte derived macrophages was significantly increased compared to the values at baseline (14). These findings are relevant to our current study and the above mentioned one. They suggest that following any immunomodulatory treatment, such as the dissociation of IL-6/IL-6R complex and/or the neutralisation of IL-6 signaling, in our study, microinvironmental changes may lead to the induction or expansion of B cells with regulatory characteristics.

B regulatory cells were reported by others to be $CD86^{high}$, $IL\text{-}10^{high}$ and $TGF\text{-}\beta^{high}$. Their incubation with Th1-induced regulatory responses, namely the reduction in Th1 activity and pro-inflammatory responses (15). In a recent study, a subset of B cells ($CD19{+}CD24^{high}CD38^{high}$) were reported to be of regulatory capacity and shown to be functionally impaired in SLE patients (16).

In another recent study, evaluating $CD25^{+}$ B cells as regulatory cells, B cell phenotype status was assessed in ANCA-related vasculitis patients during the active phase of disease *vs*. being in remission. In patients with remission, the proportion of $CD25^{+}$ B cells was increased compared to those with active disease and healthy controls, suggesting these B cells to have a regulatory role (17). In our laboratory we show that $CD19^{+}CD25^{high}CD27^{high}$ are also $IL\text{-}10^{high}$ and $TGF\text{-}\beta^{high}$ and are able to inhibit the proliferation of stimulated Th1 cells (unpublished data), suggesting this subset of cells to be both memory and of B regulatory properties (as stated in ref 9 and 17). The increase in TGF-β expression but not in IL-10 expression is thought to be due to the fact that non-stimulated B cells express very low amounts of IL-10. In association with the above phenotypic changes we show hereby the alteration in B cell activity and APC properties.

When peripheral blood B cells are activated by CD40L/IL-4, they overexpress both CD80 and HLA-DR, rendering them more efficient antigen presenting cells (APCs) than resting B cells. When HLA-DR expression on B cells was blocked by anti-HLA-DR antibody, B-APC induced activation was altered suggesting HLA-DR a crucial factor in this process (18). In this regard, it is suggested that the beneficial clinical effect of depleting B cells may relate in part to the inhibition of their APC properties (MHC-II alteration) and pro-inflammatory/activation function. The alteration of MHC-II and CD69 expression on B cells following tocilizumab treatment is highly relevant to its proposed beneficial effect by interfering with B-APC properties.

Further studies are required to establish this preliminary data in order to better establish weather this or other therapeutic regimens are indeed able to expand B regulatory properties.

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