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Concise report

Correction of abnormal B-cell subset distribution by interleukin-6 receptor blockade in polymyalgia rheumatica

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Abstract

Objectives. The aim was to study lymphocyte subsets and circulating cytokines at diagnosis of PMR and after tocilizumab monotherapy.

Methods. Eighteen untreated patients with PMR were included in a prospective study and received 3-monthly tocilizumab infusions without glucocorticoids. Lymphocyte subset distribution was assessed by flow cytometry and serum cytokines were assayed by a 34-cytokine array and ELISA, at baseline and during follow-up. Baseline data were also compared with age- and sex-matched controls.

Results. At baseline, total lymphocytes, T-cell subsets and NK cell counts were similar in patients and controls, but patients had significantly lower B-cell counts attributable to lower transitional, naïve and post-switch memory B-cell subsets. Circulating B-cell counts were positively correlated with the PMR activity score (PMR-AS) in untreated active patients at baseline, but subsequently increased to normal values while disease activity was controlled after tocilizumab therapy. Among serum cytokines, IL-6 showed the largest concentration difference between patients and controls, and the serum IL-6 concentration was correlated with baseline PMR-AS. The effects of tocilizumab on serum IL-6 concentration were heterogeneous, and the patients whose serum IL-6 decreased after tocilizumab therapy exhibited a significant increase in circulating B-cell counts.

Conclusion. In patients with PMR, B-cell lymphopenia and abnormal B-cell subset distribution are associated with disease activity and IL-6 concentration, and both are corrected by the IL-6 antagonist tocilizumab.

Key words: polymyalgia rheumatica, tocilizumab, B cells, cytokines, interleukin-6

CLINICAL SCIENCE

Rheumatology key messages

- B-Cell lymphopenia and abnormal distribution in PMR are corrected after tocilizumab therapy.
- Increased serum IL-6 in patients with PMR is correlated with disease activity.
- B-Cell monitoring in PMR might be a predictive element for the time to tocilizumab response.

Introduction

The pathophysiology of PMR remains unclear. Recent work has demonstrated low circulating B-cell counts in active PMR followed by a return to normal after CS therapy [1]. Several lines of evidence indicate that IL-6 is the pro-inflammatory cytokine that varies most widely in PMR, increasing in active disease and declining after CS therapy [2, 3], suggesting a pathophysiological role for IL-6 in the disease. Based on this evidence, tocilizumab, a mAb to the IL-6 receptor (IL-6R), was used successfully in several

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patients with refractory PMR [4]. Furthermore, in patients with newly diagnosed PMR enrolled in two open-label phase 2 studies, tocilizumab therapy induced clinical disease remission [5, 6]. The biological effects accompanying the clinical efficacy of tocilizumab are unknown.

Our aim was to compare the distribution of lymphocyte subsets and circulating concentrations of pro-inflammatory and anti-inflammatory cytokines, including IL-6, in patients with PMR, both at diagnosis and after tocilizumab therapy, as well as in age- and sex-matched controls.

Methods

Patients, controls and intervention

Eighteen patients with untreated PMR participating in the Tolerance and Efficacy of tocilizumab iN pOlymyalgia Rheumatica (TENOR) study (registered at ClinicalTrials.gov, NCT01713842) at the Brest University Hospital, France, were included. Inclusion and exclusion criteria for TENOR are described elsewhere [5]. Tocilizumab was given as three 8 mg/kg i.v. infusions, at baseline and at 4 and 8 weeks (W) later, without glucocorticoids. From W12 to W24, patients received low-dose prednisone (0.15 mg/kg/day, tapered according to a predefined schedule). Disease activity was measured by the PMR activity score (PMR-AS) [7], which includes CRP and physician and patient visual analog scale scores for disease activity, morning stiffness and shoulder elevation. The primary end point of the TENOR study was PMR-AS <10 at W12. Early response was defined as PMR-AS <10 at W4. Blood samples were collected at baseline, W2 and W12 (i.e. after tocilizumab monotherapy) then at W24 (after prednisone therapy).

We recruited 18 age- and sex-matched controls among patients visiting our rheumatology department for mechanical pain, with no infectious, auto-immune or malignant disease. All patients gave their written informed consent according to the Declaration of Helsinki. This study was approved by an ethics committee (CPP Ouest VI).

Lymphocyte subset analysis and cytokine assays

Briefly, flow cytometry with four different four-colour panels (see Flow Cytometry section of supplementary data, available at *Rheumatology* Online) was used to assess the distributions of cluster of differentiation (CD)4$^+$ and CD8$^+$ T cells, NK cells, total CD19$^+$ B cells and B-cell subsets (transitional, naïve, unswitched memory and switched memory B cells; see supplementary Fig. S1, available at *Rheumatology* Online, for the gating strategy). Serum concentrations of 34 different cytokines were assayed simultaneously using a cytokine array (Human Th1/Th2/Th17 Antibody Array – Membrane ab 169809, Abcam, Bristol, UK, lot GR181414-1; see Cytokine Array section of supplementary data and supplementary Fig. S2, available at *Rheumatology* Online) in two randomly chosen patients, at baseline and W12, as well as in two controls. Cytokines differentially expressed in patients and controls were then assayed in all patients and controls using commercial ELISA kits (see ELISA section of supplementary data, available at *Rheumatology* Online).

Statistical analysis

One patient was excluded from the longitudinal analyses because of an infection during follow-up that altered the blood-cell counts and lymphocyte subsets.

Continuous variables were described as the mean and S.E.M. and categorical variables as the number and percentage. The non-parametric Mann–Whitney *U*-test or the paired non-parametric Wilcoxon test was used, as appropriate. Correlations between continuous variables were assessed using the non-parametric Spearman test. Values of $P < 0.05$ were considered significant. Statistical analyses were performed using GraphPad Prism software v6.05 (GraphPad Software, La Jolla, CA, USA) for Windows.

Results

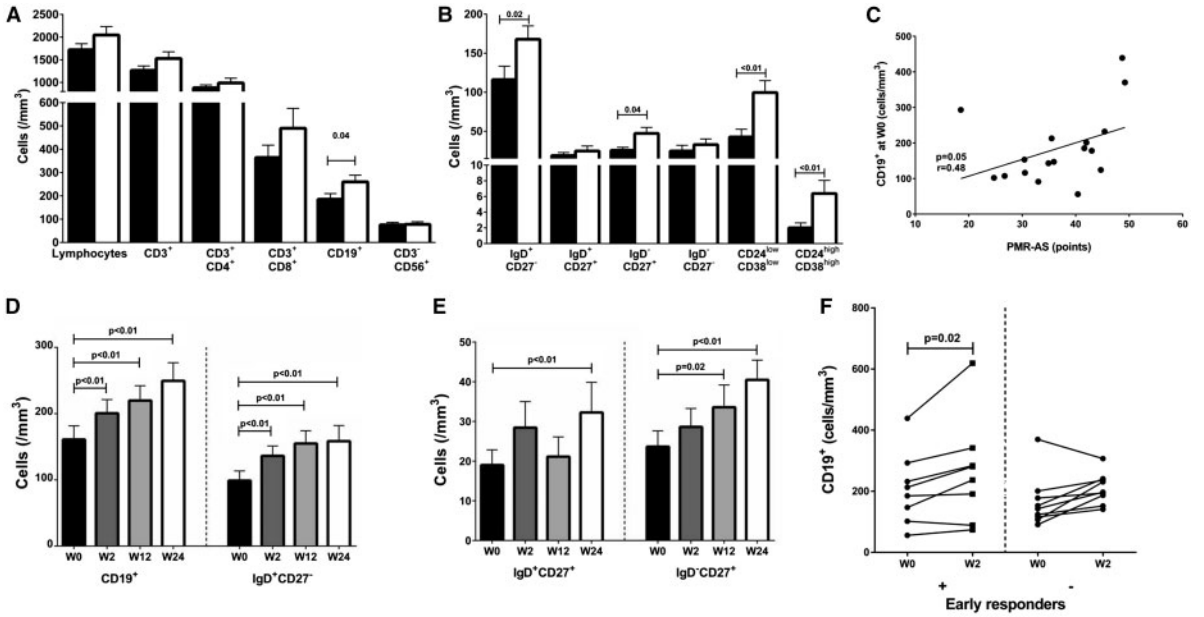
Among the 18 patients included, 7 were women (likewise for controls). Mean age at inclusion was, respectively, 68 (7) and 66 (11) years for patients and controls ($P = 0.8$). Mean BMI was, respectively, 28 (1) and 29 (1) kg/m^2 for patients and controls ($P = 0.3$). Serum concentrations of CRP were, respectively, 82 (16) and 5 (2) mg/l for patients and controls ($P < 0.01$). All patients not only fulfilled Chuang's criteria but also fulfilled the 2012 provisional ACR/EULAR criteria, received a definite diagnosis of PMR according to the evaluating physician, and underwent a systematic evaluation to rule out alternative diagnoses. After 2 years of follow-up, none of the PMR diagnosis was invalidated. In all 18 patients, PMR-AS was <10 at W12 [5]. Of note, as CRP measurements are not interpretable after tocilizumab, the detailed analysis of individual PMR-AS components showed that all clinical measures were also significantly improved during the TENOR study [5]. In eight patients, there was an early response (PMR-AS <10 at W4). No patient required CSs because of an increase of disease activity (assessed by the PMR-AS) before W12, and no patient experienced a disease flare until the end of the study (W24).

Patients with active PMR have selective B-cell lymphopenia and alterations in B-cell subsets

Total lymphocytes, CD4$^+$ and CD8$^+$ T cells and NK cells were not different between patients at baseline and controls (Fig. 1A and supplementary Table S1, available at *Rheumatology* Online). The absolute peripheral blood CD19$^+$ B-cell count was significantly lower in the patients compared with the controls, owing chiefly to significantly lower counts of IgD$^+$CD27$^-$ naïve, IgD$^-$CD27$^+$ switched memory and CD24^{low}CD38^{low} mature B cells (Fig. 1B). CD24^{high}CD38^{high} transitional B cells (Fig. 1B) were also significantly lower in patients compared with controls.

At baseline (i.e. in active untreated patients), despite this relative B-cell lymphopenia compared with controls, both total B-cell (Fig. 1C) and switched memory B-cell ($r = 0.5$, $P = 0.02$) counts were positively correlated with disease activity evaluated by the PMR-AS. This observation could be explained by a hyperinflammatory state in some patients with a dramatic increase of acute-phase

FIG. 1 B-Cell lymphopenia and B-cell subset distribution abnormalities in patients with PMR improved after tocilizumab therapy



Lymphocyte counts in patients with PMR (black bars) and controls (white bars). (**A**) Lymphocytes and subsets at baseline in patients and controls (mean ± S.D.). (**B**) B-Cell subsets in patients and controls (mean and S.E.M.). (**C**) Correlation between B-cell ($CD19^+$) count and PMR activity score (PMR-AS) at baseline (slope was calculated using linear regression). (**D** and **E**) Changes in B-cell subsets over time in the patients (mean and S.E.M.). (**F**) Changes in B-cell ($CD19^+$) counts from baseline to week 2 in early responders (PMR-AS <10 at week 4; left panel) and PMR patients without an early response (right panel).

reactants, as the serum concentration of CRP was also positively correlated with the B-cell count ($r = 0.5$, $P = 0.03$).

Tocilizumab therapy corrects B-cell subset distribution

Absolute total lymphocyte and $CD19^+$ B-cell counts increased progressively and significantly between baseline and W12 during tocilizumab monotherapy (Fig. 1D and supplementary Table S2, available at *Rheumatology* Online). This correction of the baseline B-cell lymphopenia was chiefly ascribable to increases in IgD^+CD27^- naïve B cells and IgD^-CD27^+ switched memory B cells, which were the most severely decreased subsets at baseline compared with controls (Fig. 1D and E). Other B-cell subsets also increased between baseline and W12, but to a lesser extent (supplementary Table S2, available at *Rheumatology* Online). Only patients with an early clinical response (PMR-AS <10 at W4) had significant increases in total B cells and switched memory B cells at W2 (Fig. 1F).

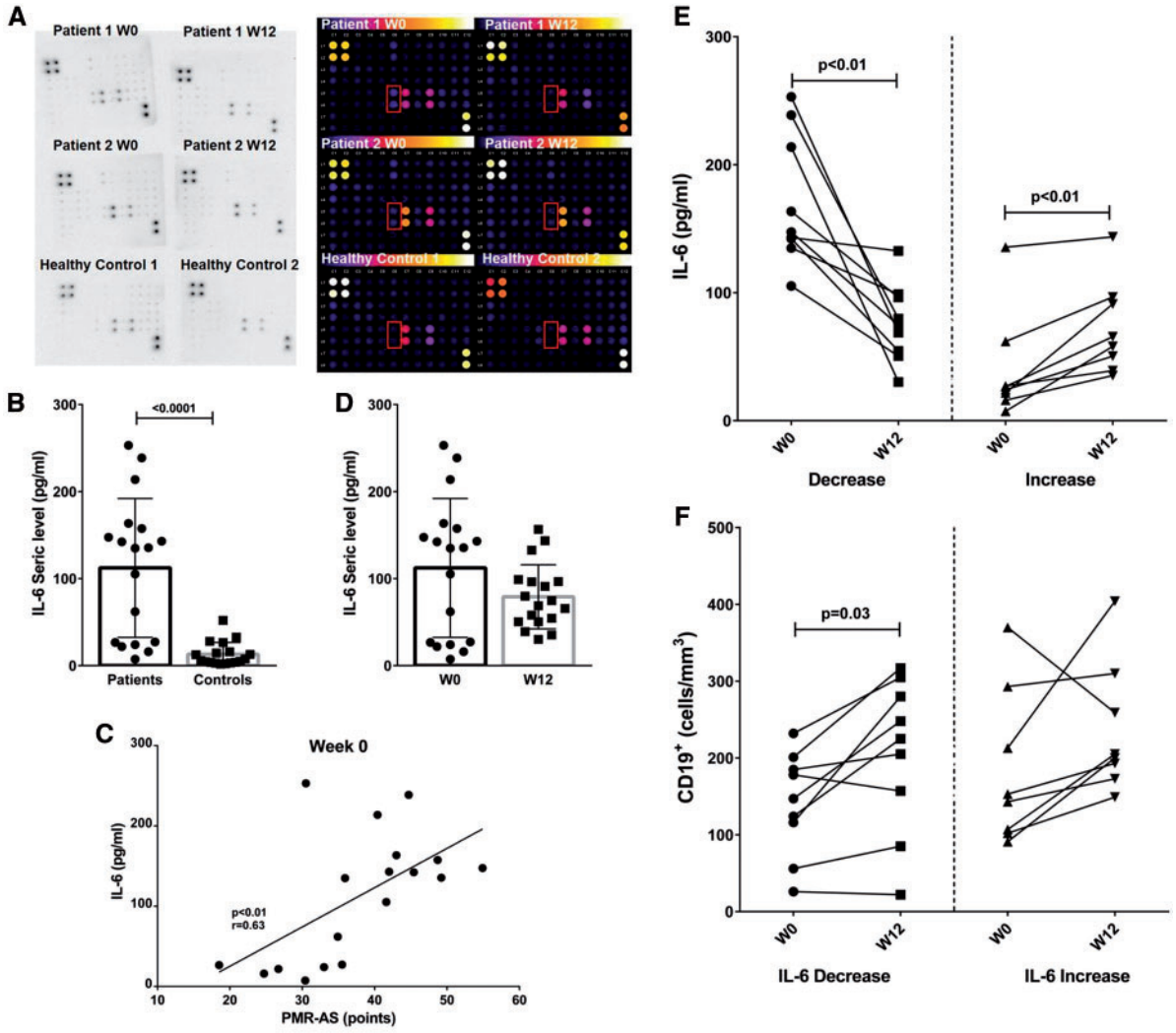
Increased serum IL-6 is correlated with disease activity in PMR patients

The cytokine array in two patients and two controls revealed a large increase in IL-6 and moderate increases in IL-1β and CD40-ligand (CD40-L) concentrations in patients at baseline compared with controls. IL-6, IL-1β, TNF-α and CD40-L concentrations in the patients were lower at W12 after tocilizumab therapy than at baseline (Fig. 2A).

As the variations were clearly largest for IL-6, we measured serum IL-6 concentrations in all controls and patients at baseline and W12, by ELISA. Serum IL-10 was also assayed as a negative marker. The mean IL-6 concentration was nearly 10-fold higher in the patients [112 (19) *vs* 13 (3) pg/ml in controls, $P < 0.01$; Fig. 2B], whereas IL-10 concentrations were similar [30 (10) and 19 (4) pg/ml, respectively; $P = 0.1$, data not shown]. The IL-6 concentration was correlated with baseline PMR-AS (Fig. 2C) and CRP concentration ($r = 0.7$, $P = 0.003$).

In the patients, the mean IL-6 concentration was not significantly lower at W12 *vs* baseline (Fig. 2D). However, the change in IL-6 concentrations from baseline to W12 varied widely across patients. In nine patients with marked baseline IL-6 elevation, IL-6 concentrations dropped between baseline and W12, from 171 (17) to 76 (10) pg/ml ($P < 0.01$). The remaining eight patients had lower baseline IL-6 concentrations, followed by a slight increase in IL-6 by W12, from 40 (15) to 72 (13) pg/ml ($P < 0.01$). Interestingly, the nine patients whose IL-6 concentrations decreased had significant increases in

FIG. 2 Serum IL-6 concentration is elevated in patients with PMR and is correlated with disease activity



(**A**) Cytokine membrane array performed in two patients at baseline and week 12 and in two controls. IL-6 spots are delineated by red lines. (**B**) Serum IL-6 concentrations determined by ELISA in patients at baseline and controls (mean ± SD). (**C**) Correlation between IL-6 and rheumatic PMR activity score (PMR-AS) at baseline ($r = 0.6$, $P = 0.005$). (**D**) Serum IL-6 concentration in patients at baseline and week 12. (**E** and **F**) Two groups of patients were defined according to IL-6 evolution: decrease *vs* increase in IL-6 serum concentration between baseline and week 12 (**E**, left and right panels, respectively). Evolution of B-cell counts between baseline and week 12 in these two groups (**F**, left and right panels, respectively).

absolute $CD19^+$ B-cell counts, from 141 (22) at baseline to 205 (33) cells/mm^3 at W24 ($P = 0.03$), and in IgD^+CD27^- naïve B-cell counts, from 81 (17) to 135 (27) cell/mm^3 ($P < 0.01$); whereas the trend for B-cell count increase we observed in the patients whose IL-6 concentrations increased was not significant (Fig. 2E and F).

Discussion

In patients with PMR, peripheral B-cell lymphopenia and abnormal B-cell subset distribution were associated with disease activity and were corrected after tocilizumab monotherapy. This effect was associated with the pro-inflammatory cytokine IL-6, because it occurred mainly in those patients whose serum IL-6 concentrations decreased after tocilizumab therapy.

Van der Geest *et al.* [1] also reported that patients with newly diagnosed active PMR had lower counts of circulating total B cells and of $CD24^{low}CD38^{low}$ mature, $CD24^{high}CD38^{high}$ transitional, IgD^+CD27^- naïve and IgD^-CD27^+ switched memory B cells compared with controls, contrasting with similar IgD^+CD27^+ unswitched memory B-cell counts. The abnormalities resolved after CS therapy. They observed a negative correlation

between ESR and B-cell concentrations after remission was induced by CSs [1], whereas we observed here a negative correlation between baseline B-cell counts and PMR-AS (and CRP) in active untreated patients, illustrating the heterogeneity of biological findings in PMR patients. Our finding of similar changes after tocilizumab monotherapy suggests that correction of the B-cell abnormalities may be attributable to a decrease in disease activity rather than to CS exposure and that inflammation and IL-6 may be responsible for the alterations in B-cell distribution. In contrast, in patients with RA, tocilizumab specifically modulated IgD^+CD27^+ unswitched memory B cells [8] and NK cells [9] and seemed to induce a decrease in memory B-cell populations [10]. These data underline the pathophysiological differences between these diseases in which B-cell subsets are disturbed [11], and the fact that most patients with RA received tocilizumab after several lines of therapies and concomitantly with MTX, whereas in the present study PMR patients received tocilizumab as a first-line monotherapy.

Several facts support a pathophysiological role for IL-6 in PMR [12]. IL-6 is increased in serum [13], muscles [14] and SF [15] from patients with PMR. A polymorphism in the IL-6 promoter is associated with PMR symptoms in patients with GCA [16]. Finally, serum IL-6 concentrations are associated with disease activity [13] and decrease after CS therapy [2]. In our study, serum IL-6 was correlated with disease activity at onset but, although all patients achieved remission after tocilizumab [5], serum IL-6 decreased only in the subgroup with the highest baseline IL-6 concentrations. Interestingly, in healthy volunteers, who received tocilizumab in a phase 1 study, and patients with RA or Castleman disease, serum IL-6 increased significantly after tocilizumab therapy, probably owing to inhibition of IL-6R-mediated clearance of IL-6 [17]. It is important to note that serum concentrations of IL-6 are different according to the disease considered; in RA, patients presented an increase in IL-6 from 58 to 93 pg/ml, and in healthy volunteers from 3 to 9 pg/ml [17]. In our study, patients with a moderately increased baseline IL-6 concentration acted in a similar manner to patients with RA, with an increase from 40 to 72 pg/ml, probably corresponding to the inhibition of IL-6 clearance as already described. But for the other patients (with very high baseline IL-6 concentrations), this increase is counterbalanced by the important decrease attributable to the control of inflammation and, probably, the decrease of IL-6 production by immune system cells.

IL-6 plays an important role in B-cell terminal differentiation [18]. It has also been reported that IL-6 could play a role in regulatory B-cell induction [19] and in the germinal centre through follicular Th cells [20]. We can assume that IL-6, by playing a role in B-cell terminal differentiation, can disturb B-cell homeostasis. Further studies are needed to unravel the precise mechanism of this interplay.

Finally, an early increase in $CD19^+$ B cells at W2 was associated with an early clinical response. Thus, the $CD19^+$ B-cell count might hold promise for predicting the time to tocilizumab response. As observed in the TENOR study, tocilizumab had a longer time to action compared with CSs, and predicting the time to response may therefore prove helpful. Given that all patients experienced an improvement of disease activity, we were not able to analyse predictive factors of the disease course during the time of follow-up.

Our study provides the first data on changes in lymphocyte populations and pro-inflammatory cytokines during tocilizumab therapy in patients with PMR, driving hypotheses regarding the pathophysiology of the disease.

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Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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