# Methotrexate reduces vaccine-specific immunoglobulin levels but not numbers of circulating antibody-producing B cells in rheumatoid arthritis after vaccination with a conjugate pneumococcal vaccine

Kapetanovic, Meliha C.; Nagel, Johanna; Nordström, Inger; Saxne, Tore; Geborek, Pierre; Rudin, Anna

# Methotrexate reduces vaccine-specific immunoglobulin levels but not numbers of circulating antibody-producing B cells in rheumatoid arthritis after vaccination with a conjugate pneumococcal vaccine

Meliha C Kapetanovic MD, PhD[1], Johanna Nagel MD [1], Inger Nordström[2] Tore Saxne MD, PhD, professor[1], Pierre Geborek MD, PhD[1], Anna Rudin MD, PhD, professor [2]

[1]**Meliha C Kapetanovic,** Department of Clinical Sciences, Lund, Section for Rheumatology, Lund University, Lund, Sweden and Skåne University Hospital, Section for Rheumatology

e-mail: meliha.c_kapetanovic@med.lu.se

[1]**Johanna Nagel,** Department of Clinical Sciences, Lund, Section for Rheumatology, Lund University, Lund, Sweden and Skåne University Hospital, Section for Rheumatology

e-mail: johanna.nagel@gmail.com

[2]**Inger Nordström,** Department of Rheumatology & Inflammation Research, Institute of Medicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

e-mail: inger.nordstrom@microbio.gu.se

[1]**Tore Saxne,** Department of Clinical Sciences, Lund, Section for Rheumatology, Lund University, Lund, Sweden and Skåne University Hospital, Section for Rheumatology,

e-mail: tore.saxne@med.lu.se

[1]**Pierre Geborek,** Department of Clinical Sciences, Lund, Section for Rheumatology, Lund University, Lund, Sweden and Skåne University Hospital, Section for Rheumatology

e-mail: pierre.geborel@med.lu.se

[2]**Anna Rudin,** Department of Rheumatology & Inflammation Research, Institute of Medicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

e-mail:anna.rudin@microbio.gu.se

**Corresponding author:**

Meliha C Kapetanovic MD, PhD

Department of Clinical Sciences, Lund, Section for Rheumatology and Skåne University Hospital, Section for Rheumatology; Lund University

Kioskgatan 5, SE-221 85 Lund, Sweden

Email: meliha c_kapetanovic@ med.lu.se

Tel ++4646/171625

## Abstract

**Background.** Treatment with methotrexate (MTX) in patients with rheumatoid arthritis (RA) leads to decreased total immunoglobulin (Ig) levels and impairs vaccine-specific IgG antibody levels following pneumococcal vaccination. The mechanisms by which MTX exerts these effects in RA are unknown. We aimed to evaluate whether MTX reduces vaccine-specific serum Ig levels and their functionality in RA patients following vaccination with pneumococcal conjugate vaccine, and if numbers of antigen-specific circulating plasmablasts are affected.

**Methods**. Ten patients with RA on MTX and 10 RA patients without disease modifying anti-rheumatic drug (DMARD) were immunized with a dose 13-valent pneumococcal conjugate vaccine (Prevenar13). Circulating plasmablasts producing total IgG and IgA as well as specific IgG and IgA against two pneumococcal capsular serotypes (6B and 23F) were enumerated using ELISPOT 6 days after vaccination. IgG levels against both these serotypes were determined with ELISA before and 4-6 weeks after vaccination. Positive antibody response was defined as $\geq$2-fold increase of pre-vaccination antibody levels. The functionality of vaccine specific antibodies to serotype 23F was evaluated by measuring their ability to opsonize bacteria using opsonophagocytic assay (OPA) in 4 randomly chosen RA patients on MTX and 4 RA patients without DMARD.

**Results.** After vaccination, RA patients on MTX showed significant increase in pre- to postvaccination antibody levels for 6B ($p<0.05$), while patients without DMARD had significant increases for both 6B and 23F ($p<0.05$ and $p<0.01$, respectively). Only 10% of RA on MTX and 40% of RA patients without DMARD showed positive post-vaccination antibody responses for both serotypes. Increased opsonizing ability after vaccination was detected in 1 of 4 RA patients on MTX and 3 of 4 patients on RA without DMARD.

However, numbers of circulating total and vaccine-specific IgG- or IgA-producing plasmablasts did not differ between RA patients with or without MTX.

**Conclusions.** MTX treatment in RA leads to reduced vaccine-specific antibody responses and their functionality compared to untreated RA following pneumococcal vaccination using polysaccharide-protein conjugate vaccine. However, since there was no reduction in numbers of circulating total or vaccine-specific antibody-producing plasmablasts after vaccination this effect is probably not due to reduced activation of B cells in lymphoid tissue.

**Keywords:** methotrexate, rheumatoid arthritis, circulating B cells, pneumococcal vaccination, antibody responses.

**Clinical trial registration: NCT02240888**

**Background**

Methotrexate (MTX) is a folic acid antagonist used for the treatment of various malignancies. Low dose MTX (≤25 mg/week) seems to have more anti-inflammatory than anti-proliferative properties and is widely used for treatment of several chronic inflammatory diseases including rheumatic diseases, psoriasis or inflammatory bowel disease [1]. The effectiveness of MTX in rheumatoid arthritis (RA) has been demonstrated in numerous studies and MTX is currently considered as an anchor disease modifying anti-rheumatic drug (DMARD) in RA [2]. However, the mechanisms by which MTX performs its disease-modifying effects are not entirely elucidated and several hypotheses have been presented of which one is suppression of B cell function (1-5).

B cells play an important role in the pathogenesis of RA. A variety of autoantibodies including antibody against citrullinated peptides (ACPA) can be found years before clinical onset of disease. The presence of ACPA and rheumatoid factor (RF) in patients with established RA are recognized markers of more severe disease. The importance of B cells in RA has re-gained attention since B cell depletion therapy has been proven efficacious in RA. The effects of MTX on the B cell compartment in patients with juvenile chronic arthritis has been studied and revealed significantly lower serum immunoglobulin (Ig) levels and proportions of transitional B cells in these patients compared to patients treated with TNF inhibitors or to patients not receiving DMARDs (6). In RA we previously reported decreased total Ig levels in patients treated with MTX compared to TNF inhibitors (7). Also, MTX treatment in systemic lupus erythematosus (SLE) patients was associated with decreased levels of total IgG, IgA and IgM compared to SLE patients without MTX treatment (8).We and others have also observed lower vaccine-specific IgG levels following pneumococcal and influenza vaccination in arthritis patients treated with MTX compared to those on TNF inhibitors, to those without DMARD and to healthy controls (9-13).

The objective of the present study was to investigate whether the effect of MTX on vaccine-specific serum immunoglobulin levels could be due to reduced activation of B cells by analyzing total and vaccine-specific circulating plasmablasts in RA patients. To this end, RA patients with or without MTX treatment were immunized with one dose of a 13-valent pneumococcal conjugate vaccine. Six days after vaccination we enumerated circulating plasmablasts producing total and vaccine-specific IgG and IgA, as well as vaccine-specific serum antibody levels before and 4-6 weeks after vaccination. As vaccine antigens in the immunoassays we chose two different pneumococcal capsular polysaccharides (6B and 23F) included in the vaccine since these two antigens are known to be associated with invasive infections (14).

**Methods**

*Study patients*

Twenty patients with RA fulfilling the ACR 1987 classification criteria for RA (15), who were regularly followed up at the Rheumatology department in Lund, Skåne University Hospital Sweden, were prospectively included in the study between December 2012 and September 2013. Ten randomly chosen RA patients treated with MTX in a stabile dose without any other DMARD and 10 RA patients not taking any DMARD were included into the study. In order to avoid the possible immunological effect of smoking or prednisolone, only non-smokers and patients not treated with systemic steroids were recruited. In addition, only patients who had not received pneumococcal vaccination within five years prior the study entry were eligible. However, none of patients had received pneumococcal vaccination previously. All participants were immunized with a single dose (0.5 ml) of 13-valent

pneumococcal conjugate vaccine (Prevenar13) administrated intramuscularly. Blood samples were taken immediately before vaccination, after 6 days and 4-6 weeks following vaccination.

*Isolation of lymphocytes*

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway). Interface PBMCs were collected, washed three times with phosphate buffer (PBS) (HyClone™, Logan, USA)  and resuspended in complete medium consisting of Iscove´s medium (Sigma- Aldrich, St. Louis, USA) supplemented with 5% fetal bovine serum (FBS; HyClone™), 50 µg/ml gentamicin (Sigma-Aldrich) and 1 mM L-glutamine (Sigma-Aldrich). Cell suspensions were kept on ice prior to being assayed for numbers of antibody-secreting cells (ASCs).

*Enumeration of antibody-secreting cells by ELISPOT*

Frequencies of total and serotype-specific ASCs using unstimulated freshly isolated PBMCs were determined by enzyme-linked immunospot assay (ELISPOT). Wells of mixed cellulose ester membrane 96 well MultiScreen HA plates (Millipore, Billerica, MA) were coated with 50 and 200 µg/ml of pneumococcal polysaccharide type 23F (Danish designation 23F) or type 6B (Danish designation 6B) (ATCC Manassas, VA, USA). For detection of total IgA and IgG, wells were coated with 5 µg/ml of affinity purified goat antibodies to the F(ab')$_2$ fragment of human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Control wells were coated with 10 µg/ml of bovine serum albumin (BSA; Sigma-Aldrich). The plates were coated overnight at 4°C and then washed four times with PBS, blocked with complete medium (Iscoves medium containing 5% FBS) and incubated at 37°C in 5% $CO_2$ for 30 minutes. The blocking was removed and different dilutions of cells in complete medium were added to the plates and incubated for 4 hours at 37°C in 5% $CO_2$. Plates were washed

three times with PBS and four times with PBS with 0.05% Tween 20 and incubated with peroxidase-conjugated affinity purified goat anti human IgA or IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:1000 in PBS -Tween with BSA at 4°C overnight. Plates were then washed four times with PBS-Tween and three times with PBS and exposed to 0.3 mg/ml of 3-amino-9-ethylcarbazole (Sigma) and 0.015% $H_2O_2$ in 0.1 M sodium acetate, pH 5.0 for 15 minutes. The plates were rinsed with tap water and dried in the dark. The spots were enumerated under magnification (x40) in a blinded fashion from at least two dilutions (16).

*Detection of vaccine-specific antibody levels in serum*

Levels of serotype-specific pneumococcal IgG to 23F and 6B were measured using the WHO standard ELISA for quantitation of human IgG antibodies specific for *S. pneumoniae* capsular polysaccharides (Pn PS ELISA), as previously described (17). Briefly, ELISA plates were coated with 1 µg Pn PS 6B or 23F. In order to diminish nonspecific binding to capsular polysaccharides, dilutions of human sera were absorbed with pneumococcal PS and then added to the ELISA plates. Goat anti-human IgG antibodies, conjugated with alkaline phosphatase, followed by addition of the substrate, nitrophenyl phosphate, were used for the detection of serotype-specific antibodies (anti-6B and anti-23F IgG). The optical density, proportional to the amount of anti-6B and anti-23F IgG present in the serum, was measured with an ELISA plate reader at 405nm. The assay was calibrated with an international reference serum that was kindly provided by Dr C. Frasch, Bethesda, MD, USA (18).The lower limit of detection was 0.01 mg/l. A vaccine antibody response was defined as a ≥2-fold increase in pre-vaccination specific antibody level.

*Opsonophagocytic assay (OPA)*

In order to evaluate the functionality of the antibodies, i.e. the ability to opsonize bacteria that leads to phagocytosis, OPA was performed on pneumococci of serotype 23F using serum collected before and 4-6 weeks after vaccination from 8 randomly chosen patients who participated in the study (4 patients with RA treated with MTX and 4 patients with RA without DMARD). As previously described, pneumococci of serotype 23F were cultured, killed by addition of glutaraldehyde, incubated for 20-30 minutes in the dark with FITC (F7250, Sigma-Aldrich, St. Louis, MO, USA) in sodium carbonate buffer, and subsequently washed three times in VBS-CaMg (veronal buffered saline with 0.15 mM Ca2+ and 0.5 mM Mg2+) (19,20). Twenty µL of FITC-labeled bacteria ($5x10^7$/mL) suspended in VBS-CaMg were incubated with 10 µL of heat-inactivated patient or control serum (prediluted 1/16 in VBS-CaMg) for 30 minutes at 37ºC. Subsequently 20 µL of baby rabbit serum (CL3441, Cedarlane, USA) was added and incubation was continued for 30 minutes at 37ºC. Polymorphonuclear neutrophils from healthy donors were preincubated with PE-labeled anti-CD66 (551480, BD Biosciences, Franklin Lakes, NJ, USA) and subsequently added to the opsonized bacteria at a final concentration of 800 cells/mL. After incubation for 30 minutes at 37 ºC, cells were analyzed with BD Accuri C6 (BD Biosciences) and numbers of cells with uptake of bacteria were calculated (20). Results were expressed as proportion (%) of polymorphonuclear cells with significant uptake of bacteria before and after vaccination. Inter- assay variation was compensated for by adjusting values to the mean value of a serum with high opsonizing ability, included in each analysis. A negative control consisting of bacteria preincubated only with BSA and no serum was also included in each analysis.

*Statistical analysis*

Demographic, disease and treatment characteristics of RA patients with MTX and RA patients not receiving any DMARD were compared using Mann-Whitney U-test (continuous variables) and Chi-square test (categorical variables). Pre- and post-vaccination geometric mean antibody levels (GML) were calculated from logarithmic transformed values. Differences in pre- and post-vaccination antibody levels for each serotype were compared using Wilcoxon test, while differences in antibody levels between treatment groups were calculated using Mann-Whitney U test. Proportions of patients responding to the vaccine were compared using Chi-square test. A p-value $\leq 0.05$ was regarded as statistically significant.

**Results**

Demographic, disease and treatment characteristics of the study population are summarized in Table 1. RA patients on MTX treatment had significantly longer disease duration, lower number of swollen and tender joints, lower ESR and lower functional score according to HAQ compared to RA patients without DMARD treatment. No previous invasive pneumococcal infection was reported among patients who participated in the study.

Patients on MTX treatment showed a significant increase in specific antibody levels only for serotype 6B 4-6 weeks after vaccination with the pneumococcal conjugate vaccine (p<0.05) (Figure 1 A and B). Of note, only one MTX treated patients had an obvious pre- to post-vaccination increase in antibody levels for serotype 6B while remaining 9 patients showed slight or no increase at all (Figure 1 A). RA patients without DMARD exhibited significant increase in post-vaccination levels for both serotypes (p<0.05 and p<0.01, respectively) (Figure 1 C and D). In accordance, post-vaccination antibody levels against serotype 23F were significantly lower in RA patients receiving MTX compared to RA patients without DMARD (p=0.007; Table 2), while post-vaccination antibody levels for serotype 6B did not

differ significantly between the groups (Table 2). The proportions of RA patients on MTX with positive vaccine-specific antibody response for serotype 6B, 23F and 6B+23F are shown in Figure 2. Only one out of 10 RA patients on MTX was vaccine responders to both serotype 6B and 23F, whereas four out of 10 RA patients without DMARD were vaccine responders.

We also evaluated opsonophagocytic activity on pneumococcal serotype 23 in sera of 8 randomly chosen patients. Only 1 out of 4 (25%) RA patients on MTX developed an increase in proportion of polymorphonuclear cells with significant opsonophagocytic activity (i.e. uptake of bacteria) after vaccination compared to before vaccination, while 3 of 4 (75%) RA patients without DMARD developed an increase in OPA. This is shown in more details in Additional file 1. The limited number of samples precluded statistical calculations.

When we enumerated the Ig-producing cells six days after vaccination we found that the numbers of total IgG- and IgA-producing B cells did not differ in RA patients receiving MTX compared to RA patients without DMARD (Table 3). Likewise, there were no differences in numbers of IgG antibody-producing cells specific for serotypes 6B and 23F between the two RA groups (Table 3, Figure 3). No significant correlation was found between numbers of antibody secreting cells and antibody titers for any of serotypes. Of note, vaccine-specific circulating antibody-producing cells of IgA isotype were absent or few (Table 3).

In summary, vaccination with a pneumococcal conjugate vaccine in RA patients resulted in reduced levels of vaccine-specific serum antibody response and an impaired ability to opsonize bacteria in those treated with MTX compared to those without DMARD treatment, whereas there was no difference between the groups in numbers of circulating vaccine-specific plasmablasts, at least not to the polysaccharide antigens 6B and 23F.

**Discussion**

In the present study we aimed at exploring the impact of MTX treatment in RA on B cell function both as reflected by vaccine-specific serum antibody response, the functionality of vaccine-specific antibodies and by the induction of vaccine-specific plasmablasts to a pneumococcal conjugate vaccine. We found that 4-6 weeks after vaccination of RA patients treated with MTX had lower vaccine-specific antibody levels to pneumococcal capsular polysaccharides 6B and 23F and an impaired ability to opsonize bacteria than RA patients not receiving DMARDs. Our group previously reported impaired antibody responses to the same pneumococcal serotypes in MTX treated patients with RA compared to spondyloarthopathy patients not receiving DMARDs, but RA patients without active treatment were not included in that study (7). To our knowledge, there are no other published data comparing the immunogenicity and protective functionality of pneumococcal conjugate vaccine in RA patients treated with MTX or without DMARD. However, a number of studies reported diminished antibody response in RA patients on MTX compared to RA without MTX or healthy controls immunized with pneumococcal polysaccharide vaccine (10-12). In addition, antibody responses to influenza vaccination in MTX-treated patients with RA was reduced compared to RA controls without DMARD confirming the negative impact of MTX on a T cell dependent antibody response (13). Of note, in this study RA patients without MTX had higher pre-vaccination antibody levels for both serotypes compared to MTX treated patients. Since none of the patients had previously been vaccinated against pneumococci, this probably reflects the effect of MTX on antibodies acquired after previous pneumococcal infections.

Suppression of B and T cell proliferation by MTX may be due to inhibition of folic acid-dependent steps in *de novo* synthesis of purines and pyrimidines, which results in lower production of a variety of T cell cytokines important in the T cell stimulation of B cells (1-5). Immunization of mice with a T cell dependent antigen showed that MTX treatment decreased the numbers of B cells in the spleen and had a strong inhibitory effect on antigen-specific IgG

levels (21). The conjugation of pneumococcal capsular polysaccharides to a protein carrier (i.e. CRM 197 diphtheria protein) used in pneumococcal conjugate vaccine provides foreign protein antigens to the immune system, which is required for a T cell dependent antibody response. After vaccination naïve immature B cells from the bone marrow differentiate either into short-lived plasma cells or long-lived plasma cells and memory cells in response to the polysaccharide-protein antigens (22). In the germinal center of the lymph node and spleen, antigen-specific activated T-helper cells provide signals to the B cells that undergo clonal proliferation and differentiation into plasmablasts, which secrete antigen-specific antibodies and exit the lymph node into the circulation (22). In the present study we found that numbers of vaccine-specific plasmablasts in the peripheral blood 6 days after immunization with pneumococcal conjugate vaccination were not lower in RA patients treated with MTX compared with RA patients without any DMARD treatment. Recently it was found that MTX treatment of children with juvenile chronic arthritis resulted in lower numbers of circulating transitional/naive B cells compared with those not treated with MTX, but not in reduction of other maturational stages of B cells. This indicates that early stages of B cell development might be more susceptible for MTX inhibition than recently activated mature B cells (6). Another possibility is that MTX affects the numbers of long-lived plasmablasts in the bone marrow.

There are some limitations to this study. Firstly, the number of patients included in the study was low, but in spite of this we found significant differences in vaccine responses between the MTX-treated and the untreated groups. Secondly, we analyzed the antibody response to 2 of 13 serotypes included in the vaccine as we assumed that antibody response to these two serotypes would reflect the response to other vaccine antigens. The MTX treated patients responded to only serotype 6B indicating differential immunogenicity of the pneumococcal capsular polysaccharides although the difference was driven by only one patient having an

obvious increase in pre- to postvaccination antibody levels to that serotype. Secondly, there were some significant differences in baseline disease characteristics between the RA treatment groups as RA without DMARDs included mainly patients with recently diagnosed RA with higher disease activity. However, the strength of the study are that only patients with diagnosed with RA were included and that smoking and other anti-rheumatic treatments including corticosteroids and anti-CD20 antibodies were not permitted.

In conclusion, MTX treatment is associated with decreased levels of vaccine-specific immunoglobulins and functionality of these antibodies but not numbers of antibody-producing B cells in RA patients after vaccination with a conjugate pneumococcal vaccine. Further studies on the B cells and plasma cells in the bone marrow compartment are needed to enhance our understanding of mechanism of action of low-dose MTX in RA with regard to the reduced vaccine-specific antibody responses.

**List of abbreviations**

RA = rheumatoid arthritis; MTX= methotrehate; DMARD= disease modifying anti-rheumatic drug; Ig= immunoglobulin; ELISA= enzyme-linked immunosorbent assay; ELSPOT= enzyme-linked immunospot assay; OPA= opsonophagocytis assay

**Declarations**

*Ethics approval and consent to participate*

Ethical approval was obtained from the Regional Ethical Review Board at Lund University, Lund, Sweden (Dnr 2011/341). Oral and written consent was obtained from all participants before the inclusion in the study.

*Availability of data and material*

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

*Author contributions*

The study was conceived by MCK, TS, PG and AR. MCK, JN and AR wrote the manuscript and all authors have revised it for important intellectual content. IN has set up and performed the ELISOPT analyses and constructed tables and figures. All authors have approved the final version of the manuscript for submission.

**References:**

1. Wessels JA, Huizinga TW, Guchelaar HJ. Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis. Rheumatology (Oxford). 2008;47:249–255.

2. Rosenthal GJ, Weigand GW, Germolec DR, Blank JA, Luster MI. Suppression of B cell function by methotrexate and trimetrexate. Evidence for inhibition of purine biosynthesis as a major mechanism of action. J Immunol. 1988;141:410–16.

3. Cribbs AP, Kennedy A, Penn H, Amjadi P, Green P, Read JE, et al. Methotrexate Restores Regulatory T Cell Function Through Demethylation of the FoxP3 Upstream Enhancer in Patients With Rheumatoid Arthritis. Arthritis Rheumatol. 2015 May;67:1182-92.

4. Chan ES, Cronstein BN. Mechanisms of action of methotrexate. Bull Hosp Jt Dis (2013). 2013;71 Suppl 1:S5-8. Review.

5. Thomas S, Fisher KH, Snowden JA, Danson SJ, Brown S, Zeidler MP. Methotrexate is a JAK/STAT pathway inhibitor. PLoS One. 2015;10:e0130078.

6. Glaesener S, Quách TD, Onken N, Weller-Heinemann F, Dressler F, Huppertz HI, et al. Distinct effects of methotrexate and etanercept on the B cell compartment in patients with juvenile idiopathic arthritis. Arthritis Rheumatol. 2014;66:2590-600.

7. Kapetanovic MC, Roseman C, Jönsson J, Truedsson L, Saxne T, Geborek P. Methotrexate but not TNF-blockers reduces antibody response following pneumococcal vaccination using 7-valent conjugate pneumococcal vaccine in adult patients with arthritis. Arthritis Rheum 2011;63:3723-32.

8. Miyawaki S, Nishiyama S, Aita T, Yoshinaga Y. The effect of methotrexate on improving serological abnormalities of patients with systemic lupus erythematosus. Mod Rheumatol. 2013;23:659-66.

9. Roseman C, Truedsson L, Kapetanovic MC. The effect of smoking and alcohol consumption on markers of systemic inflammation, immunoglobulin levels and immune response following pneumococcal vaccination in patients with arthritis. Arthritis Res Ther. 2012;14:R170

10. Kapetanovic MC, Saxne T, Sjöholm A, Truedsson L, Jönsson G, Geborek P. Influence of methotrexate, TNF-blocker and prednisolone on antibody responses to

pneumococcal polysacharide vaccine in patients with rheumatoid arthritis. Rheumatology 2006;45:106-111.

11. Mori S, Ueki Y, Akeda Y, Hirakata N, Oribe M, Shiohira Y, et al. Pneumococcal polysaccharide vaccination in rheumatoid arthritis patients receiving tocilizumab therapy. Ann Rheum Dis. 2013;72:1362-6.

12. Winthrop KL, Silverfield J, Racewicz A, Neal J, Lee EB, Hrycaj P, et al. The effect of tofacitinib on pneumococcal and influenza vaccine responses in rheumatoid arthritis. Ann Rheum Dis. 2016;75:687-95.

13. Kapetanovic MC, Saxne T, Mörner A, Aktas T, Kristensen LE, Geborek P. Impact of anti-rheumatic treatment on immunogenicity of pandemic H1N1 influenza vaccine in patients with arthritis. Arthritis Res Ther. 2014;16:R2.

14. Henriques Normark B, Kalin M, Örtqrist A, Akerlund T, Liljequist BO, Hedlund J, et al. Dynamics of penicillin-susceptible clones in invasive pneumococcal disease. J Infect Dis 2001;184:861–9.

15. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.

16. Czerkinsky CC, Nilsson LA, Nygren H, Ouchterlony O, Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J Immunol Methods. 1984;65:109-21.

17. World Health Organization. Training manual for enzyme linked immunosorbent assay for the quantification of Streptococcus pneumoniae serotype specific IgG (Pn PS ELISA): a guide to procedures for qualification of materials and analysis of assay performance. Accessed at

http://www.vaccine.uab.edu/ELISA%20Protocol.pdf.

18. Quataert SA, Rittenhouse-Olson K, Kirch CS, Hu B, Secor S, Strong N, et al. Assignment of weight-based antibody units for 13 serotypes to a human antipneumococcal standard reference serum, lot 89-S(f). Clin Diagn Lab Immunol 2004;11:1064-9.

19. Martinez J.E, Romero-Steiner S, Pilishvili T, Barnard S, Schinsky J, Goldblatt D, et al. A Flow Cytometric Opsonophagocytic Assay for Measurement of Functional Antibodies Elicited after Vaccination with the 23-Valent Pneumococcal Polysaccharide Vaccine. Clin Diagn Lab Immunol.1999;:581-6.

20. Jönsson G, Lood C, Gullstrand B, Holmström E, Selander B, Braconier JH, et al. Vaccination against encapsulated bacteria in hereditary C2 deficiency results in antibody response and opsonization due to antibody-dependent complement activation. Clin Immunol. 2012;144: 214-27.

21. Salinas-Carmona MC, Perez LI, Galan K, Vazquez AV. Immunosuppressive drugs have different effect on B lymphocyte subsets and IgM antibody production in immunized BALB/c mice. Autoimmunity. 2009;42:537-44.

22. Yoshida T, Mei H, Dörner T, Hiepe F, Radbruch A, Fillatreau S et al. Memory B and memory plasma cells. Immunol Rev.2010;237:117-39.

**Table** 1. Demographic, disease and treatment characteristics of the study population

**Table 2**. Pre- and postvaccination geometric mean levels of vaccine-specific anti-6B and anti-23F IgG in serum

**Table 3.** Postvaccination number of IgG and IgA total and specific antibody-secreting cells

**Figure legends**

**Figure 1**. Pre- and postvaccination antibody levels (logarithmic transformed values) for serotype 6B and 23F before and 4-6 weeks after vaccination with a 13-valent pneumococcal conjugate vaccine in RA patients on MTX (A and B) and RA without DMARD (C and D). *p<0.05 and **p<0.01 (Wilcoxon test). A p-value ≤0.05 was regarded as statistically significant.

**Figure 2**. Proportions of vaccine responders defined as patients with ≥2-fold increase in pre- to postvaccination specific antibody levels for serotype 6B, for serotype 23F and for 6B and 23F combined. ***P=0.007 (Chi-squared test).

**Figure 3**. Frequency of anti-6B IgG (A) and anti-23F IgG (B) specific antibody-secreting cells (ASC) in peripheral blood from RA patients on Methotrexate (MTX) compared with RA patients without any treatment (No DMARD) 6 days after vaccination with a 13-valent pneumococcal conjugate vaccine. Results are expressed as the median number of ASC per $10^5$ peripheral blood mononuclear cells (PBMC). Ns= nonsignificant, P≥ 0.05 (Mann-Whitney U-test)

**Additional file 1**. Opsonophagocytic assay (OPA) performed on serotype 23F among 8 randomly chosen RA patients. The figure shows the proportion (%) of polymorphonuclear cells with significant uptake of bacteria before vaccination and 4-6 weeks after vaccination

Table 1. Demographic, disease and treatment characteristics of the study population

| | Rheumatoid arthritis patients | | |
| | Receiving MTX (n=10) | No DMARD (n=10) | *P* value |
|---|---|---|---|
| Age; years, median (range) | 67.4 (39.1-78.6) | 67.3 (38.6-86.7) | 0.912[a] |
| Sex (% female) | 70 | 80 | 0.606[b] |
| Disease duration at vaccination; years, median (range) | 8 (1-39) | 0 (0-12) | **0.035**[a] |
| ACPA positive (%) | 70 | 50 | 0.317[b] |
| RF positive (%) | 80 | 80 | 1[b] |
| SJC at vaccination (0-28) median (range) | 0 (0-6) | 6.0 (0-17) | **0.011**[a] |
| TJC at vaccination (0-28), median (range) | 0 (0-6) | 3.5 (0-15) | **0.007**[a] |
| Erosive disease (%) | 30 | 40 | 0.639[b] |
| CRP at vaccination; mg/L, median (range) | 3.3 (0-11) | 5.8 (0-48) | 0.436[b] |
| ESR at vaccination; mm/hour, median (range) | 15.5 (5-42) | 29 (8-46) | **0.028**[a] |
| HAQ at vaccination (0-3), median (range) | 0.3 (0-0.55) | 0.6 (0.1-1.9) | **0.030**[a] |
| MTX dose; mg/week, median (range) | 20 (12.5-25) | ----- | |

[a]Mann-Whitney U-test; [b] Chi-squared test;

ACPA=antibodies against citrullinated peptides, RF= rheumatoid factor; SJC= swollen joint count; TJC=tender joint count; HAQ =health assessment questionnaire; MTX=methotrexate
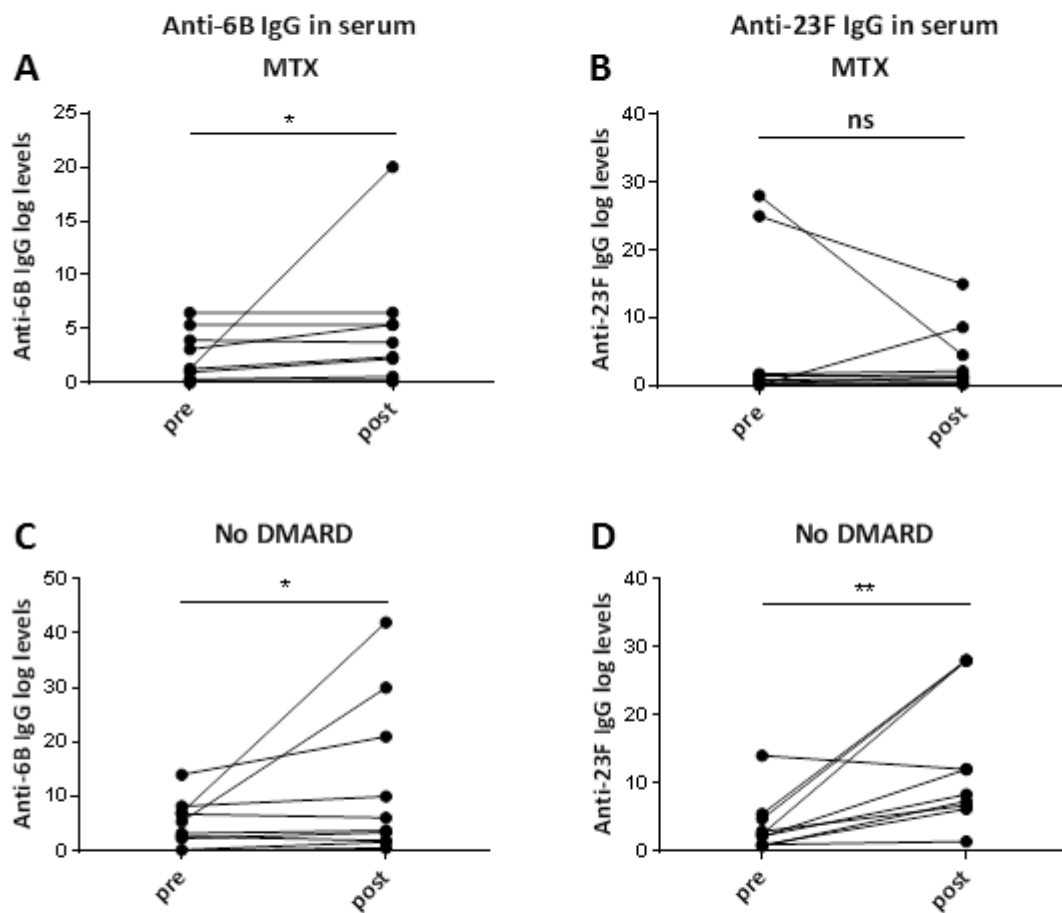
**Figure 1**



Anti-6B IgG in serum

Anti-23F IgG in serum

Table 2. Pre- and postvaccination geometric mean levels of vaccine-specific anti-6B and anti-23F IgG in serum

| | Rheumatoid arthritis patients | |
| --- | --- | --- |
| | Receiving MTX (n=10) | No DMARD (n=10) |
| Pre anti-6B IgG; g/L (95% CI)[a] | 1.3 (0.5-32) | 2.5 (0.7-8.3) |
| Post anti-6B IgG; g/L (95% CI) | 2.1 (0.7-6.4) | 5.7 (2.1-15.4) |
| Pre anti-23F IgG; g/L (95% CI) | 1.0 (0.3-4.1) | 2.4 (1.2-4.7) |
| Post anti-23F IgG; g/L (95% CI) | **1.7 (0.6-4.4)** | **10.1 (5.2-19.5)**[b] |

[a]GML (geometric mean levels); [b]p=0.007 (Mann-Whitney U test)

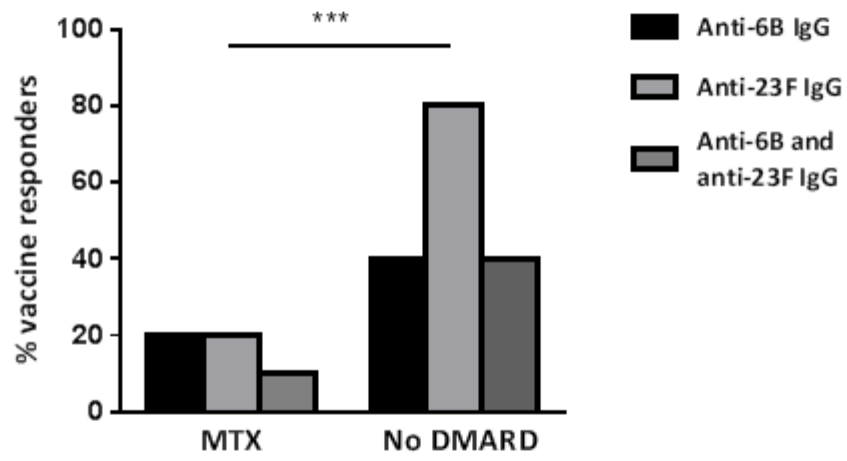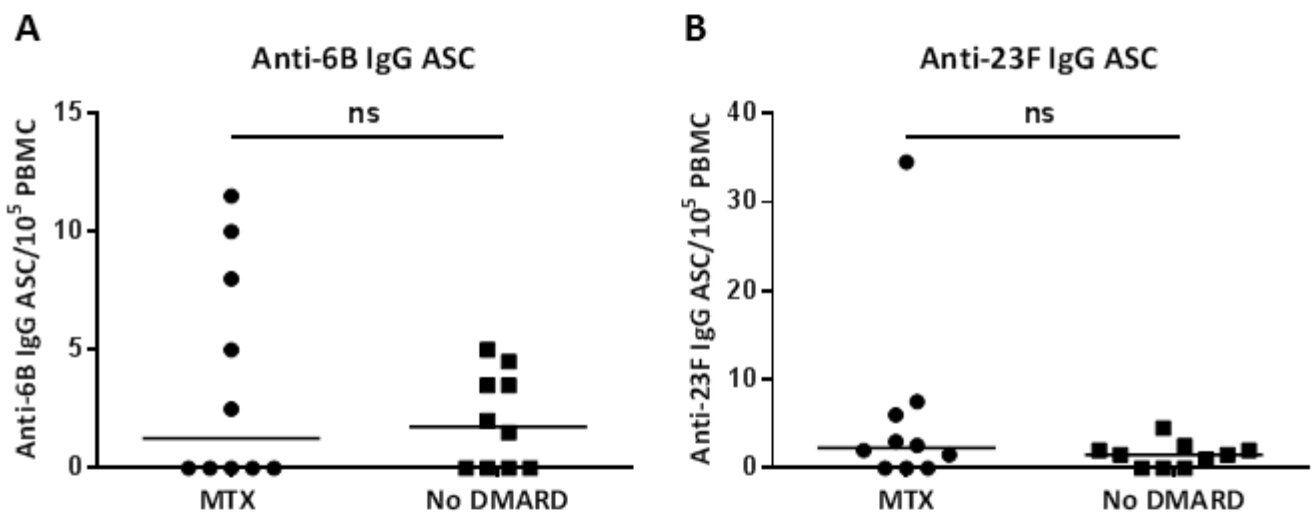MTX=Methotrexate, DMARD=disease modifying anti-rheumatic drugs

**Figure 2**

Table 3. Postvaccination number of IgG and IgA total and specific antibody-secreting cells

| Number of Ig-producing cells/$1\times10^5$ PBMCs | Rheumatoid arthritis patients | | |
|---|---|---|---|
| | Receiving MTX (n=10) | No DMARD (n=10) | *P* value[b] |
| Total IgG | 195 (10-840)[a] | 170 (70-560) | 0.78 |
| Anti-6B IgG | 1.25 (0-11.5) | 1.75 (0-5) | 0.68 |
| Anti-23F IgG | 2.3 (0-34.5) | 1.5 (0-4.5) | 0.28 |
| Total IgA | 65 (5-800) | 100 (20-650) | 0.38 |
| Anti-6B IgA | 0 (0-2.5) | 0 (0-2.1) | 0.61 |
| Anti-23F IgA | 0 (0-1) | 0.5 (0-5.5) | 0.20 |

[a] Median (range) [b] Mann-Whitney U test

**Figure 3**

**A**

Anti-6B IgG ASC

ns

Anti-6B IgG ASC/$10^5$ PBMC

MTX    No DMARD

**B**

Anti-23F IgG ASC

ns

Anti-23F IgG ASC/$10^5$ PBMC

MTX    No DMARD

# Additional file 1

## Opsonophagocytic activity on pneumococcal serotype 23F