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Impact of tofacitinib treatment on human B-cells *in vitro* and *in vivo*

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ABSTRACT

B-cells are pivotal to the pathogenesis of rheumatoid arthritis and tofacitinib, a JAK inhibitor, is effective and safe in its treatment. Tofacitinib interferes with signal transduction via cytokine receptors using the common γ-chain. Despite extensive data on T-lymphocytes, the impact of tofacitinib on B-lymphocytes is poorly understood. In this study we assessed the effect of tofacitinib on B-lymphocyte differentiation and function. Tofacitinib treatment strongly impaired *in vitro* plasmablast development, immunoglobulin secretion and induction of B-cell fate determining transcription factors, *Blimp-1*, *Xbp-1*, and *IRF-4*, in naïve B-cells. Interestingly, class switch and activation-induced cytidine deaminase (*AICDA*) induction was only slightly reduced in activated naïve B-cells. The effect of tofacitinib on plasmablast formation, immunoglobulin secretion and proliferation was less profound, when peripheral blood B-cells, including not only naïve but also memory B-cells, were stimulated. In line with these *in vitro* results, the relative distribution of B-cell populations remained stable in tofacitinib treated patients. Nevertheless, a temporary increase in absolute B-cell numbers was observed 6–8 weeks after start of treatment. In addition, B-cells isolated from tofacitinib treated patients responded rapidly to *in vitro* activation. We demonstrate that tofacitinib has a direct impact on human naïve B-lymphocytes, independently from its effect on T-lymphocytes, by impairing their development into plasmablasts and immunoglobulin secretion. The major effect of tofacitinib on naïve B-lymphocyte development points to the potential inability of tofacitinib-treated patients to respond to novel antigens, and suggests planning vaccination strategies prior to tofacitinib treatment.

1. Introduction

Rheumatoid arthritis (RA) is a multifactorial, immune-mediated disease. Biological DMARDs successfully control RA disease activity by inhibiting co-stimulation, depleting B-lymphocytes or targeting pro-inflammatory cytokines [1]. Cytokine signalling is mediated by cytokine receptors via activation of janus kinases (JAKs) and the recruitment of corresponding signal transducers and activator of transcription (STATs) [2]. JAK3 and STAT1, 4, and 6 are highly expressed in the synovium of RA patients [3]. Tofacitinib inhibits signalling via cytokine receptors mainly associated with JAK3 and/or JAK1 [4–6] and has successfully been introduced into the armamentarium of RA therapy [6–11]. JAK3 is predominantly expressed in hematopoietic cells and is essential for signal transduction via the common γ-chain (γ_c), therefore, it is necessary for activation, differentiation and homeostasis of lymphocytes [12]. Furthermore, tofacitinib impairs the differentiation of $CD4^+$ T helper cells [5]. Despite extensive data on the effect of tofacitinib on T-lymphocytes, the knowledge on the effects of tofacitinib on B-cells is scarce. In RA, B-cells accumulate in the synovium, where they can even form germinal centers and differentiate into plasma cells [13,14]. In addition, they secrete chemokines and cytokines

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and may function as antigen-presenting cells amplifying and worsening synovial T-cell-mediated inflammation [15]. Cytokines can activate JAK-STAT signalling pathways in B-cells [16–18]. Indeed, triggering of the IL-21 receptor, a γ_c cytokine-receptor, induces the differentiation of B-cells into plasma cells, and B-cell-intrinsic signalling through IL-21 receptor and STAT3 is necessary for long-lived antibody responses in humans [16]. Therefore, it can be assumed that tofacitinib via interfering with IL-21 signaling will have a direct, T-cell independent effect on B-cell differentiation and function. In line with these data, genetic defects in the γ_c or *JAK3* in severe combined immunodeficiency (SCID) result in T-cell deficiency, but also in impairment of B-cell memory generation and antibody production [12,19,20]. We studied the direct effect of tofacitinib on naïve and total blood B-lymphocyte survival, activation, proliferation and differentiation *in vitro*. We examined antibody class switch and secretion. In addition we assessed the absolute numbers and distribution of B-lymphocyte subpopulations in patients during tofacitinib treatment as well as the dynamics of *in vitro* activation of B-cells isolated from tofacitinib treated patients. The results of our experiments implicate beneficial effects of tofacitinib in predominantly B-cell-driven autoimmune diseases and may impact on vaccination strategies in patients treated with tofacitinib.

2. Materials and methods

2.1. Patients selection and ethics

Umbilical cord blood was obtained after caesarean delivery upon signed informed consent under approval of the University Freiburg Ethics Committee (174/13). Buffy coats were purchased from the blood bank of the University Medical Center Freiburg (approval of the University Freiburg Ethics Committee: 147/15). Thirteen consecutive patients with polyarthritis (mean age 58.3, anti-CCP positive 25%) were included into the study. Tofacitinib was used as monotherapy in 4 patients and combined with conventional synthetic DMARDs in 9 patients. Twelve out of 13 patients responded to therapy (as defined by disease activity score DAS and CRP-concentrations) after 3–6 months of tofacitinib treatment. No correlation between the CCP antibody concentrations and the B cell subset distribution was observed. Blood samples of the patients were collected prior to the start of tofacitinib treatment and/or during follow-up.

The studies were approved by the respective institutional review boards of the University of Basel (protocol number EKNZ 2014/051) and the University Medical Center Freiburg (191/11, 46/04). Informed consent was obtained from the patients as well as the healthy donors. All experiments were carried out in accordance with the Declaration of Helsinki.

2.2. In vitro B-cell cultivation

Tofacitinib was provided by Pfizer (Pfizer Inc, Peaback, USA) and dissolved in sterile water by sonication. CD40L and IL-21 were prepared as described previously [21]. Magnetically sorted B-cells from cord blood or buffy coat (1.5×10^5 cells/ml) were stimulated with soluble trimeric CD40L [22] and IL-21 or with CpG (ODN2009, Apara Biosciences) in the absence or presence of scalar doses (10, 100, 300, and 1000 nM) of tofacitinib. Cord blood contained in average 5–20% of B cells, of which transitional T1 cells were about 6–25%, and T2 cells were about 10–29%. The remaining cells were naïve (40–70%), no switched memory cells were detected *ex vivo* (Supplementary Fig. 1) [23]. B-cells were cultivated for 3–9 days in Iscoves' medium supplemented with 10% FCS, insulin, apotransferrin, non-essential amino acids, glutamine, and reduced glutathione as described earlier [21]. Starting the culture with equal numbers of B-cells, the absolute numbers of B-cells and plasmablasts were determined by counting the events in the CD19/live or the $CD27^{high}CD38^{high}$ gate by flow cytometry.

2.3. Flow cytometry

Phenotypes of B-cells were determined by flow cytometry with the following antibodies: CD19 APC-H7, CD38 PE-Cy7 (all BD), CD27 PerCP-Cy5.5, IgD PE (BioLegend), IgM DyLight 649, IgG DyLight 649, and IgA DyLight 649 (Jackson ImmunoResearch). Dead cell exclusion was performed by DAPI staining. B-cell proliferation was monitored by CFSE (Molecular Probes) labelling, following manufacturer's instructions. In the CD19/viable lymphocyte gate naïve B-cells were defined as $CD27^{-}IgD^{+}$, marginal zone B-cells were defined as $CD27^{+}IgD^{+}$, switched memory B-cells were defined as $CD27^{+}IgD^{-}$, transitional B-cells were defined as $CD38^{high}IgM^{high}$, and plasmablast were defined as $CD38^{high}CD27^{high}$. Absolute counts of B-lymphocytes and subpopulations were calculated on the base of the patients' lymphocyte counts at day of analysis.

2.4. Determination of immunoglobulin concentrations

Immunoglobulins (Ig) in culture supernatants were measured by ELISA. In brief, 96-well plates (Nunc Maxisorp) were coated with anti-human Ig mix (Jackson Immuno Research) in bicarbonate buffer. Bound Ig were detected with alkaline phosphatase-conjugated anti-human IgM, IgG or IgA (Jackson ImmunoResearch), and developed with *p*-nitrophenyl phosphate (Sigma-Aldrich) in DEA buffer. Optical density was measured at 405 nm. The parallel OD detection of a progressive dilution of a protein Ig standard (N Protein Standard SL; Siemens) starting with a concentration of 100 ng/ml up to 1,25 ng/ml ensured the calculation of the amount of each Ig in the supernatant. The experimental control was the stimulation with CD40L and IL-21 of the B cells, known to induce class switch and immunoglobulin secretion. The immunoglobulin secretion of tofacitinib treated activated B cells *in vitro* was compared to untreated activated B cells.

2.5. Quantitative PCR

RNA was extracted using TRIZOL reagent (Invitrogen) and quantified with the NanoDrop 2000 (Thermo Scientific). cDNA synthesis was performed with 200 ng RNA using SuperScript III reverse transcriptase (Invitrogen) and random hexamer primers (Amersham Pharmacia Biotech). Quantitative PCR was performed using the TaqMan® Gene Expression Master Mix (Applied Biosystems) and a StepOnePlus Real Time PCR system (Applied Biosystem). qPCR reaction was performed with previously published primer/probe sets: *Blimp-1*, *Xbp-1*, and *IRF-4*, *AICDA* (activation-induced cytidine deaminase) [21] (all purchased from Eurogentec). The following amplification program was used: 2 min at 50 °C and 10 min at 95 °C followed by 45 cycles consisting of 15 s at 95 °C and 1 min at 58 °C. Relative expression was calculated using the $2^{-\Delta Cq}$ method with all mRNA levels standardized to the reference *RPLPO* (Applied Biosystems). Standard deviation of gene expression in qPCR experiments was calculated as described [24].

3. Results

3.1. Tofacitinib severely impairs plasmablast development and immunoglobulin secretion from naïve B-cells

Naïve follicular B-cells encountering antigens in the germinal center develop into plasmablasts via activating signals mediated by