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# The effect of JAK inhibitors baricitinib and tofacitinib on Th17 cells, regulatory T cells and the Th17/$T_{reg}$ balance in patients with rheumatoid arthritis

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Das dieser Arbeit zugrunde liegende Biomaterial (Blutproben) wurde ohne meine Mitarbeit durch Angestellte der rheumatologischen Ambulanz der Klinik für Innere Medizin I der Uniklinik Köln gewonnen. Die klinische Untersuchung zur Erhebung des DAS28 (Disease Activity Score 28) wurde von PD Dr. Kofler in der rheumatologischen Ambulanz durchgeführt. Die Studie wurde durch die Ethikkommission beraten und unter der Nummer 13-091 genehmigt.

Die in dieser Arbeit angegebenen Experimente und Methoden sind nach entsprechender Anleitung durch Frau Meyer von mir selbst ausgeführt worden. Ebenso verhält es sich mit der statistischen Auswertung der gewonnenen Daten.
Die in der Arbeit genutzten Laborwerte habe ich selbst dem Patienteninformationssystem *ORBIS* der Uniklinik Köln entnommen.

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# Index

## ABBREVIATIONS

| | |
|---|---|
| ACPA | Anti-citrullinated protein antibodies |
| ACR | American college of Rheumatology |
| AHR | Aryl hydrocarbon receptor |
| Ang1 | Angiopoietin 1 |
| APC | Allophycocyanin |
| AS | Ancylosing spondylitis |
| B2M | Beta 2- macroglobulin |
| Cbl-b | Cbl Proto-Oncogene B |
| CD | Cluster of differentiation |
| CIA | Collagen-induced arthritis |
| CRP | c-reactive-protein |
| DAS28 | Disease Activity Score 28 |
| DC | Dendritic cell |
| DGRh | Deutsche Gesellschaft für Rheumatologie |
| DMARDs | Disease Modifying Anti-Rheumatic Drugs |
| DNA | Deoxyribonucleic acid |
| EAE | Experimental autoimmune encephalomyelitis |
| EDTA | Ethylendiamintetraacetat |
| EULAR | European League Against Rheumatism |
| FACS | Fluorescence-activated cell scanning |
| FITC | Fluorescein isothiocyanate |
| FGF | Fibroblast growth factor |
| FoxP3 | Forkhead-Box-Protein 3 |
| F-SC | Forward Scatter |
| G-CFS | Granulocyte-Colony Stimulating Factor |
| GM-CFS | Granulocyte macrophage colony-stimulating factor |
| HC | Healthy Control |
| HLA-DRB1 | Human Leukocyte Antigen – DR isotype beta chain 1 |
| IFN-γ | Interferon gamma |
| IL- | Interleukin |
| JAK | Janus Kinase |
| MACE | Major adverse cardiovascular events |
| MACS | Magnetic cell separation |
| MCP | Metacarpophalangeal Joint |
| MHC | Major histocompatibility complex |

| | |
|---|---|
| MMP | Matrix metalloproteinases |
| MTP | Metatarsophalangeal joints |
| mRNA | Messenger Ribonucleic Acid |
| MRI | Magnetic resonance imaging |
| n | Number |
| NSAID | Non-steroidal anti-inflammatory drug |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PE | Phycoerythrin |
| PerCP-CY5.5 | Peridinin-Chlorophyll-Protein complex – Cyanine5.5 |
| PI3K | Phosphoinositid-3-Kinase |
| PMA | Phorbol-12-myristat-13-acetat |
| PIP | Proximal interphalangeal joints |
| PsA | Psoriatic arthritis |
| RA | Rheumatoid arthritis |
| RNA | Ribonucleic acid |
| RORC | RAR-related orphan receptor C |
| rpm | Revolutions per minute |
| SLE | Systemic lupus erythematodes |
| STAT | Signal transducer and activator of transcription proteins |
| SS-C | Sideward Scatter |
| Tbx21 | T-box 21 |
| TGFβ | Transforming growth factor beta |
| Th | T helper |
| TNFα | Tumor necrosis factor alpha |
| TRAF6 | TNF Receptor Associated Factor 6 |
| VEGF | Vascular Endothelial Growth Factor |

# 1. Zusammenfassung

Die rheumatoide Arthritis (RA) ist eine chronische Autoimmunerkrankung, die durch entzündliche Gelenkdestruktionen zu schmerzhaften Funktionseinschränkungen und fortschreitender Behinderung führt. Begleitend sind häufig extraartikuläre Manifestationen an Nieren, Lunge, Herz-Kreislaufsystem, Haut und Augen zu beobachten, die mit erhöhtem kardiovaskulärem Risiko und reduzierter Lebenserwartung assoziiert sind. Die Standardtherapie mit Methotrexat ist grundsätzlich effektiv, zeigt aber bei einigen Patient:innen ein unzureichendes Therapieansprechen und außerdem ausgeprägte, häufig therpielimitierende Nebenwirkungen. Zur gezielteren Behandlung mit hoher klinischer Effektivität bei tolerablen Nebenwirkungen wurden vor kurzem JAK Inhibitoren als sogenannte targeted Disease Modifying Anti-Rheumatic Drugs (DMARDs) zur Therapie der RA zugelassen. JAK Inhibitoren gehören zur Klasse der Tyrosinkinase-Inhibitoren und wirken als Immunmodulatoren, die durch Blockade des intrazellulären JAK/STAT Signalweges die Differenzierung und Aktivierung verschiedener Immunzellen beeinflussen.

Eine große Bedeutung in der Pathogenese der RA wird insbesondere einem Ungleichgewicht von stark pro-inflammatorischen Th17-Zellen und immuno-regulatorischen T-Zellen zugeschrieben. Studien konnten sowohl signifikante Erhöhungen von Th17-Zellen als auch reduzierte Zellzahl und Funktionseinschränkungen von regulatorischen T-Zellen ($T_{reg}$) in RA erkrankten Patient:innen nachweisen, sodass das Th17/$T_{reg}$ Gleichgewicht zugunsten von Th17-Zellen verschoben ist. Vor diesem Hintergrund stellte sich die Frage, welchen molekularen Effekt die neu zugelassenen JAK Inhibitoren auf Th17-Zellen, regulatorische T-Zellen und deren Gleichgewicht haben - insbesondere, ob sie in der Lage sind, das immunologische Gleichgewicht wiederherzustellen. Außerdem untersucht die Studie, ob es Unterschiede zwischen den zwei zugelassenen JAK Inhibitoren gibt, da Tofacitinib vor allem JAK1 und JAK3 hemmt, während Baricitinib vor allem JAK1 und JAK2 hemmt, und dadurch in der Theorie verschiedene Signalkaskaden beeinflusst werden. Begleitend stellte sich die Frage, ob JAK Inhibitoren auch Einfluss auf die Genexpression der einzelnen Januskinasen haben oder ‚nur' den nachgeschalteten Signalweg hemmen.

In die Studie eingeschlossen wurden Patient:innen der rheumatologischen Ambulanz der Uniklinik Köln mit diagnostizierter RA, sowie eine Kontrollgruppe aus weiteren Ambulanz-Patient:innen mit ähnlichen epidemiologischen Merkmalen ohne autoimmune Grunderkrankung. Die RA Patient:innen wurden je nach Behandlung in 4 Untergruppen eingeteilt: unbehandelt, Baricitinib-behandelt, Tofacitinib-behandelt und Methotrexat-behandelt. Wir entnahmen den Patient:innen periphere Blutproben, isolierten $CD4^+$ T-Zellen und analysierten diese mittels Durchflusszytometrie und PCR zur Darstellung der oben genannten T-Zell Subgruppen und zur Bestimmung der Expression von Januskinasen und Zell-typischen Transkriptionsfaktoren.

Bei Patient:innen mit unbehandelter RA zeigten sich signifikant erhöhte Expressionen (3.50% ± 0.63; *p*= 0.0012) des Th17 assoziierten Schlüsselzytokins IL-17, welches für die chronische Entzündungsreaktion verantwortlich gemacht wird. Durch die Behandlung mit Baricitinib, Tofacitinib und MTX konnten die IL-17 Expressionen effektiv gesenkt werden und wieder das Expressionsniveau von Gesunden erreichen. Die erhobenen Daten zeigen außerdem, dass die Anzahl regulatorischer T-Zellen in $CD4^+$ T Zellen in unbehandelten RA Patient:innen (5.33% ± 0.14) im Vergleich zu Gesunden (8.93% ± 0.67; *p*= 0.0005) signifikant reduziert ist und bestätigen damit das pathologische Ungleichgewicht zwischen Th17-Zellen und $T_{reg}$-Zellen. Interessanterweise führte die Behandlung mit JAK Inhibitoren Baricitinib und Tofacitinib nicht zu einer Steigerung der regulatorischen T-Zellen, während MTX-behandelte Patient:innen eine partiell wiederhergestellte $T_{reg}$-Zellzahl aufweisen (6.98% ± 0.61). Bezogen auf den Th17/$T_{reg}$ Quotienten sind JAK Inhibitoren daher insgesamt nur teilweise in der Lage, das zelluläre Gleichgewicht wiederherzustellen – eine Erkenntnis, die bei der Weiterentwicklung von Therapiestrategien möglicherweise von Bedeutung sein kann.

Die Analyse der Genexpression der Januskinasen JAK1, JAK2, JAK3 und TYK2 zeigte zunächst, dass erkrankte Patient:innen im Vergleich zu Gesunden keine erhöhte JAK Expression aufweisen. Soweit im Rahmen dieser Studie zu beurteilen, scheint auch die Behandlung mit JAK Inhibitoren keinen Einfluss auf die Expression von Januskinasen zu haben, sodass deren Wirkung am ehesten auf die reduzierte Januskinase-Aktivität, und nicht auf deren geringere Expression zurückzuführen ist. Trotz der unterschiedlichen Affinität der beiden JAK Inhibitoren zu den vier bekannten Janus Kinasen JAK1, JAK2, JAK3 und TYK2 konnte in dieser Studie kein Unterschied zwischen der Behandlung mit Baricitinib und Tofacitinib in Bezug auf die JAK/STAT assoziierte T-Zell Differenzierung festgestellt werden.

Basierend auf den Ergebnissen der Doktorarbeit lässt sich insbesondere das immunologische Ungleichgewicht der pro-inflammatorischen Th17-Zellen und der immuno-regulatorischen $T_{reg}$ -Zellen in Patient:innen mit RA bestätigen. Zudem zeigt der fehlende Effekt der untersuchten JAK Inhibitoren auf $T_{reg}$ -Zellen, dass die Therapie hinsichtlich der Wiederherstellung des Th17/$T_{reg}$ Gleichgewichtes weiteres Potenzial zur Verbesserung hat. Zum einen könnten Kombinationstherapien mit low-dose IL-2 einen neuen Ansatz bilden, da IL-2 über STAT5 die Proliferation und Differenzierung von regulatorischen T-Zellen fördert. Zum anderen wurden selektivere JAK Inhibitoren, wie zum Beispiel JAK1 Inhibitor Upadacitinib oder TYK2 Inhibitor BMS-986165 entwickelt, um noch gezieltere Wirkung zu erzeugen. Durch weitere Forschung mit selektiven JAK Inhibitoren kann außerdem ein tieferes Verständnis über die Januskinase-assoziierten Signalwege gewonnen werden, welches für die Verbesserung der Therapie der RA und anderen Autoimmunerkrankungen von großer Bedeutung ist.

## 2. Summary

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease that leads to irreversible structural joint damage, progressive disability and severe systemic complications including pulmonary, cardiac, hematological and vascular disorders. It affects around 1% of the full age population and is the most common form of inflammatory arthritis worldwide. The first-line therapy with the immunosuppressant methotrexate is generally efficient and effective, but provokes a big range of adverse effects and, at times, shows inadequate treatment response. In order to improve the therapeutic management of Rheumatoid Arthritis, the JAK inhibitors baricitinib and tofacitinib - a new class of so called targeted Disease Modifying Anti-Rheumatic Drugs (DMARDs) - recently achieved RA approval, passing clinical phase III trials with high effectiveness and tolerable adverse effects. Due to their oral application, they have an additional advantage in clinical use. JAK inhibitors are immunomodulators that target cytokine receptor associated janus kinases and thereby affect the JAK/STAT-mediated differentiation and activation of different immune cells.

Considering the pathogenesis of RA, especially the immunologic imbalances of pro-inflammatory Th17 cells and immunosuppressive regulatory T ($T_{reg}$) cells seem to play a pivotal role in the development of disease. In RA patients, increased frequencies of Th17 cells are found while $T_{reg}$ cells are impaired, leading to a defective Th17/$T_{reg}$ ratio in favor of Th17 cells. With that in mind, this study was constituted in order to investigate the molecular effect of baricitinib and tofacitinib on JAK/STAT-mediated activation and differentiation of Th17 cells and $T_{reg}$ cells in patients with RA. We particularly focused the question, if JAK inhibitors are able to restore the impaired Th17/$T_{reg}$ balance. Moreover, we aimed to detect differences in the molecular effects of baricitinib and tofacitinib taking into account that they target different janus kinases. Baricitinib is known as a JAK1/JAK2 inhibitor, while tofacitinib was designed to inhibit primarily JAK1 and JAK3. Last, this study analyzed the effect of JAK inhibitor treatment on janus kinase expression levels, investigating if reduced JAK activation also affects its expression.

The study was performed with patients of the rheumatologic ambulance of the University Hospital of Cologne, including patients with diagnosed rheumatoid arthritis and a ‘healthy’ control group without rheumatic diseases but similar epidemiological features. The RA patients were subclassified in 4 groups according to their treatment: baricitinib-treated, tofacitinib-treated, methotrexate-treated and untreated. Peripheral blood samples were taken, $CD4^+$ T cells were isolated and then analyzed via flowcytometry and PCR in order to display the frequency of the different T cell subsets [%] in relation to $CD4^+$ T cells on the one hand, and to evaluate the expression of janus kinases and T cell associated key-transcriptions factors on the other hand.

Patients with untreated rheumatoid arthritis showed significantly increased expression levels (3.50% ± 0.63; *p*= 0.0012) of the Th17 cell associated key cytokine IL-17 compared to healthy controls (1.31% ± 0.18), promoting a chronic inflammatory state. We showed that both treatments, MTX and the JAK inhibitors baricitinib and tofacitinib, were highly effective in the reduction of IL-17 expression achieving similar expression levels as healthy controls. The study also revealed that the frequency of regulatory T cells is significantly reduced in untreated RA patients (5.33% ± 0.14) compared to healthy controls (8.93% ± 0.67; *p*= 0.0005), confirming the pathologic imbalance of Th17 cells and $T_{reg}$ cells in disease. Interestingly, neither baricitinib nor tofacitinib was able to restore the impaired regulatory T cells, in contrast to MTX, which at least partially enhanced them (6.98% ± 0.61). With regard to the Th17/$T_{reg}$ ratio, this study concludes that JAK inhibitors only partially restore the immunologic balance via their effect on Th17 cells. This finding might form a background for prospective improvements of JAK inhibitor treatment in the therapeutic management of rheumatoid arthritis and other inflammatory diseases.

The analyzed gene expression levels of the janus kinases JAK1, JAK2, JAK3 and TYK2 slightly indicate that JAK expression is not increased in patients with rheumatoid arthritis compared to healthy controls. Further, it can be assumed, that the mechanism by which JAK inhibitors affect JAK-induced downstream signaling is probably rather related to reduced JAK activation than to reduced JAK expression. But further studies with larger data sets need to be addressed to make a precise statement on the molecular effect of JAK inhibitors on JAK gene expressions. Although baricitinib and tofacitinib target different janus kinases, this study did not show any differences in the effect of baricitinib and tofacitinib on JAK/STAT-mediated differentiation and activation of the analyzed T cells.

Taken together, the most intriguing finding of this study was the demonstration of low $T_{reg}$ cell numbers in peripheral blood after treatment with baricitinib and tofacitinib, which leads in total to a partially restored Th17/$T_{reg}$ balance that is mainly achieved by a strong therapeutic effect on pathogenic Th17 cells. Based on these results, we presume that novel therapeutic strategies with the ability to fully restore the Th17/$T_{reg}$ balance might bring additional benefit in the treatment of rheumatoid arthritis. One approach could be the combination of JAK inhibitors with low-dose IL-2, considering that IL-2 receptor downstream signaling induces $T_{reg}$ cell differentiation. This might recover $T_{reg}$ cell frequencies and could thereby contribute to immune homeostasis. Another promising approach is the development of more selective JAK inhibitors such as a JAK 1 inhibitor upadacitinib and TYK2 inhibitor BMS-986165. These selective JAK inhibitors and its research will further improve our understanding of janus kinase mediated downstream signaling and bring new insights into immunologic processes of the pathogenesis of rheumatoid arthritis and other autoimmune diseases.

# 3. Introduction

Rheumatoid arthritis (RA) is the most common rheumatic autoimmune diseases and affects about 1% of the adult population in Europe leading to progressive disability and reduced quality of life. Therefore, intensive research is being conducted in order to develop new treatment strategies and to improve disease management and patient's outcome. Recently, first janus kinase (JAK) inhibitors were approved for the treatment of RA and were recommended in case that conventional synthetic DMARDs fail. These JAK inhibitors, named baricitinib and tofacitinib, showed promising results and significant improvement of disease activity. It is known that JAK inhibitors block the signaling pathway of various cytokines. However, the molecular mechanisms by which they ameliorate disease activity are not completely understood.

## 3.1 Rheumatoid Arthritis

### 3.1.1. Definition, epidemiology and clinical features

Rheumatoid arthritis is a systemic inflammatory autoimmune disease that leads to irreversible structural joint damage, progressive disability and a range of systemic complications with reduced life expectancy[1,2]. According to the German Society for Rheumatology (Deutsche Gesellschaft für Rheumatologie, DGRh)[3], RA affects approximately 1% of the full age population in Germany and is worldwide the most common form of inflammatory arthritis. Although RA can occur at any age, people aged 50-70 years are mostly affected and the ratio between female and male patients is 2,5:1. The general risk of disease onset dramatically increases with genetic susceptibility and age progression, but is also linked to cigarette smoking, dietary factors and infections[4-6]. Aside from high disease-associated medical costs, rheumatoid arthritis generates relevant socioeconomic burden caused by functional disability and reduced work capacity [7].

The clinical appearance is predominated by symmetrically affected peripheral joints of wrist, fingers and ankle, whereby metacarpophalangeal (MCP) joints, proximal interphalangeal (PIP) joints and metatarsophalangeal (MTP) joints are mainly involved. Bigger joints like elbow, knee, shoulder and hip are affected less often[8]. Typical initial symptoms include joint pain, morning stiffness and swelling accompanied by general symptoms like low-grade fever, rheumatoid nodules under the skin, loss of appetite and mental-health problems as depression and fatigue[1]. The clinical course of RA is extremely variable and appears in a range from mild, self-limited arthritis to rapid progressive inflammation with extra-articular involvement

becoming manifest in ocular, pulmonary, cardiac, gastrointestinal, hematological, and vascular disorders such as anemia, vasculitis, parenchymal lung diseases, keratoconjunctivitis, pericarditis or gastritis[9,10] (figure 1). Severe RA is further associated with profound morbidity and mortality and patients have a two times increased risk for cardiovascular diseases, heart attack and stroke[11,12].

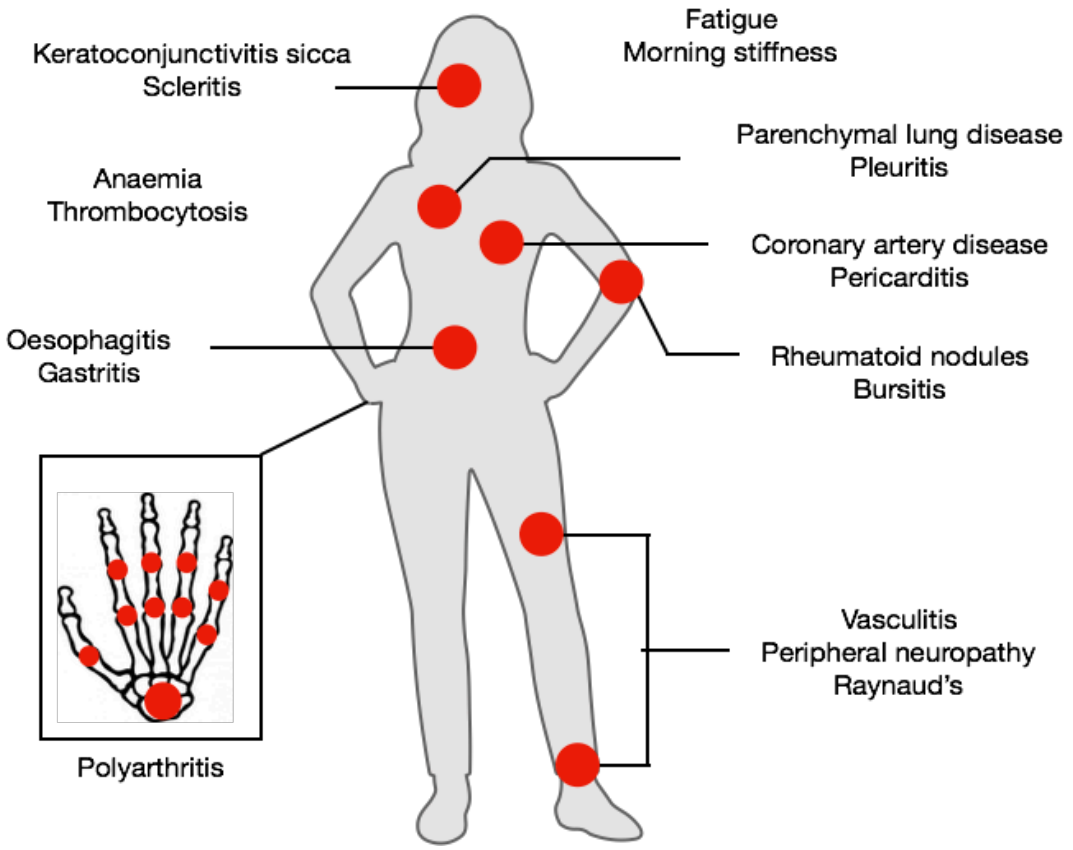


**Figure 1: Clinical features of rheumatoid arthritis.** Patients primarily suffer from joint damage and pain in wrist and fingers, especially in metacarpophalangeal joints, proximal interphalangeal joints and metatarsophalangeal joints. Further, patients can show severe extra-articular manifestations including ocular, pulmonary, cardiac, gastrointestinal, hematological, and vascular disorders. (Figure adapted from Gulati M.[9])

### 3.1.2. Diagnosis of rheumatoid arthritis

The most common classification system for rheumatoid arthritis was defined by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) and incorporates four different items: joint involvement, serology, acute-phase reactants and the duration of signs and symptoms[13]. The items are assessed by a scoring system with a maximum score of 10 points among all four items. The diagnosis of RA can be confirmed reaching an overall score of ≥ 6 points (table1). Joint involvement as a major parameter for RA

is assessed by clinical appearance focusing on swollen and painful joints. Joint destruction can be assessed by standard x-ray imaging scans to evaluate disease progress and to classify the severity of joint damage. The acute phase reactants erythrocyte sedimentation rate (ESR) and C- Reactive Protein (CRP) are used to evaluate the extend of systemic inflammation and disease activity. The analysis of the serologic status is based on the expression of rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA). Both, RF and ACPA are strong indicators for RA, but do not confirm the diagnosis as its expression is also seen in other rheumatic diseases. Depending on the serology, RA is sub-classified in seropositive and seronegative RA. Finally, the self-reported symptom duration is taken into account, considering a symptom persistence of ≥ 6 weeks as risk factor for a chronic course of disease.

| | | |
|---|---|---|
| Joint destruction | • 1 large joint<br>• 2-10 large joints<br>• 1-3 small joints<br>• 4-10 small joints<br>• >10 joints (at least one small joint) | • 0<br>• 1<br>• 2<br>• 3<br>• 5 |
| Serology | • negative RF **and** negative ACPA<br>• Low positive RF **or** low positive ACPA<br>• High positive RF **or** high positive ACPA | • 0<br>• 2<br>• 3 |
| Symptom duration | • < 6 weeks<br>• > 6 weeks | • 0<br>• 1 |
| Acute phase reactants | • normal CRP **and** normal ESR<br>• Abnormal CRP **or** abnormal ESR | • 0<br>• 1 |
| | | **≥ 6 = definite RA** |

**Table 1: ACR/EULAR Rheumatoid arthritis classification criteria.** Criteria for the diagnosis of rheumatoid arthritis defined by the American College of Rheumatology and the European League Against Rheumatism (EULAR) in 2010. Assessment of the 4 items joint destruction, serology, symptom duration and acute phase reactants with a total score ≥ 6 confirms the diagnosis of RA.

### 3.1.3. Pathogenesis of rheumatoid arthritis

The pathogenesis of RA is heterogeneous, and its complex mechanisms are not fully understood. Genetic susceptibility, environmental factors, epigenetic modifications, infections and dysregulated immune pathways were described to play an important role in the

development of disease[14]. All of these factors contribute by different mechanisms to a loss of tolerance and thereby promote aberrant activation of innate and adaptive immune response.

The genetic susceptibility is mainly ascribed to variants in human leukocyte antigen (HLA) - DRB1 beta chain, a major histocompatibility class II receptor molecule that is encoded by *HLA-DRB1* gene[15]. HLA-DRB1 is expressed at the surface of antigen presenting cells (APCs) and plays a central role in the immune system by presenting extracellular proteins to T helper cells. Revealing the high polymorphism of the *HLA-DRB1* gene, a long-time established hypothesis emphasized a strong link between distinct *HLA-DRB1* risk alleles and the development of rheumatoid arthritis[16]. These risk alleles – most popular the HLA-DRB1*04 (HLA-DR4) subtypes – encode a certain amino acid sequence motif that is known as the 'shared epitope' and found in about 80% of RA patients[17,18]. Acting as a potent immune-stimulatory ligand, the shared epitope in DRB1 was shown to polarize T cell differentiation towards pathogenic Th17 cells while suppressing the differentiation into regulatory T cells[19]. This crucial role in T cell differentiation is further strongly associated with ACPA positive RA and implicates immune dysregulation with highly increased risk of disease. However, HLA-DRB1 genotyping is not useful in clinical practice as the risk allele is rare and the association is rather weak.

From all environmental factors, cigarette smoking was reported to be the strongest external trigger of rheumatoid arthritis[20]. Smoking promotes the generation of RA associated autoantibodies by posttranslational modification and direct interaction with genetic components[21]. Furthermore, increased levels of pro-inflammatory cytokines were related to cigarette smoking and contribute to an impaired immunologic homeostasis promoting the pathogenesis of RA[22]. Among smoking, unhealthy lifestyle factors, caffeine, air pollution, medications and dietary factors like high intake of sodium and sugar as well as red meats have also been variably linked to the risk of developing rheumatoid arthritis[5,6]. Recently, the development and perpetuation of RA was also associated with alterations in the intestinal microbiota, supposing that mucosal dysbiosis facilitates the activation of immunomodulatory cells and thereby contributes to local and systemic inflammation[23]. Although, the molecular interaction between gut microbiota and the immune system still need to be studied, mucosal dysbiosis seems to gain importance not only in the pathogenesis of RA, but also in other autoimmune and neuroinflammatory diseases such as systemic lupus erythematosus, spondylarthritis, multiple sclerosis or Alzheimer's disease[24,25].

Considering the immunomodulatory effects of infectious agents such as EBV, CMV, parvovirus B19, and *E.coli*, they have been postulated to be associated with acute arthralgia, arthritis and rheumatoid arthritis by the formation of immune complexes and the induction of antibodies like

the rheumatoid factor (RF)[14]. Results from a recent meta-analysis about evidence of the association between viral exposure and development of RA, found a higher risk for RA after ParvoB19, HCV and possibly EBV infection, while CMV and HBV seem not to be associated with an increased risk to develop RA[26]. Within the Covid-19 pandemic, first case reports were published describing onset of rheumatoid arthritis immediately after SARS-Cov-2 infection[27,28].

## 3.2 The innate and adaptive immune system in the development of rheumatoid arthritis

RA is characterized by chronic inflammation based on abnormal innate, cellular and humoral immunity[29]. Several immunologic mechanisms were shown to play a pivotal role in the onset of RA including joint-associated micro-environmental changes, dysregulated T cell activation as well as impaired homeostasis and cell migration. In the following, most important immunologic functions and RA-related dysfunctions of the immune system are shortly pictured giving an overview on the mode of action of Th17 cells and regulatory T cells. Further, the role of janus kinase/signal transducers and activators of transcription (JAK/STAT)-mediated signaling and its potential therapeutic target is shortly described.

### 3.2.1. The innate immune system in rheumatoid arthritis

The innate immune system protects the organism through first line barriers and non-specific defense mechanisms from pathogens and infected cells. It is mainly represented by macrophages, dendritic cells (DCs), mast cells, and the complement system and forms the initial and rapid immune response that is based on recognition of microbial pathogens, antigen presentation and release of inflammatory cytokines and chemokines. These cytokines and chemokines further promote the activation of the adaptive immune system and induce the recruitment of other immune cells to sites of infection[30].

In rheumatoid arthritis, the number of circulating monocytes is decreased, whereas the number of activated monocytes/macrophages in the synovium of affected joints is significantly increased[31]. This indicates a remarkable shift of monocytes from peripheral blood towards inflamed joints, where the monocytes differentiate into highly activated synovial macrophages. Activated macrophages produce a range of inflammatory cytokines such as TNF-$\alpha$, IL-1$\beta$, IL-6 and thereby promote the activation of effector T cells and induce inflammation and destructions of the joints[32]. The macrophages further secrete pro-angiogenic factors such as Matrix-metalloproteinases (MMPs), chemokines (CC, CXC) and growth factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and angiopoietin

(Ang1/Ang2), which are all involved in neovascularization and consequently facilitate excessive cell migration and progressive inflammation[33].

Beside the macrophages, also the dendritic cells are strongly involved in the pathogenesis of rheumatoid arthritis based on their important role in differentiation and activation of T cells. DCs guide T cell priming in lymph nodes and thereby shape the generation of central and peripheral tolerance including the deletion of auto-reactive T cells. Referring to rheumatoid arthritis, it is assumed that inappropriate T cell development might be a strong trigger for disease onset[34]. Underlining the importance of well working DCs in the development of balanced and adequate immune responses, a mouse model study showed that DC depletion causes rapidly developed autoimmunity with formation of autoantibodies and increased numbers of $CD4^+$ T cells[35]. Further, it has been shown that DCs from patients with RA produce higher amounts of pro-inflammatory cytokines IL-6 and IL-23 than DCs from healthy controls. Via production of these inflammatory cytokines, they additionally contribute to the activation of effector T cells and persistent inflammation[36]. This strong interaction of macrophages/DCs and effector T cells, already indicates the complexity of the innate and adaptive immune system and underlines the variety of immunologic mechanisms that can be involved in immune dysregulation.

### 3.2.2. The adaptive immune system in rheumatoid arthritis

The adaptive immune system with its highly specialized B and T lymphocytes enables rapid and specific immune response to particular pathogens in order to protect the organism from infections and toxic molecules. Their major function is to recognize and eliminate these foreign pathogens, while self-antigens need to be spared. The capacity to distinguish self and foreign antigens derives from selections in the thymus and is essential to prevent autoimmunity. Passing the maturation process, both, T cells and B cells undergo a series of gene segment rearrangements that form a huge diversity in antigen receptors, but also implicate the risk of generating autoreactive T- and B- lymphocytes[37]. While B lymphocytes are able to mark and to neutralize antigens through the production of specific antibodies, cytotoxic T lymphocytes can directly eliminate cells presenting antigens and T helper cells produce cytokines that activate macrophages and other immune cells[38]. Apart from primary immune response, another important compound is the establishment of an immunological memory, which enables even more rapid and effective immune response to previously encountered pathogens. Antigen-specific memory B cells have, in contrast to naïve B cells, an increased affinity to particular antigens and higher levels of MHC class II molecules, which leads to expanded cell function at lower antigen doses[37].

In RA, on the one hand, defective B and T cell maturing was shown to be responsible for the generation of autoreactive immune cells characterized by impaired tolerance with immune response to self-molecules[39,40]. On the other hand, the number of highly activated B and T cells in peripheral blood of patients with RA is increased, resulting in high secretion levels of inflammatory cytokines and chemokines[41]. The high levels of inflammatory cytokines combined with macrophage-mediated angiogenesis and synovial tissue formation strongly promote the recruitment of other pro-inflammatory T cells and further increase the secretion of inflammatory cytokines which again promote the activation of effector T cells. Thus, once established, autoimmune dysregulation seems to be a self-reinforcing system that is characterized by a positive feedback loop of enhanced inflammatory cells and inflammatory cytokines. But nevertheless, the trigger for the initial onset of disease and activation, proliferation and migration of inflammatory cells still needs to be figured out.

Recently, RA was mainly considered as a T cell mediated disease with focus on T cell associated dysfunctions like dysregulated release of inflammatory cytokines, aberrant signaling and impaired cell function. In particular the four different T-helper cell subsets Th1, Th2, Th17, and regulatory T cells ($T_{reg}$) are involved in immunological response, so that dysfunction of these cells was mentioned as main driver for chronic inflammation and disease activity[41]. The differentiation from naïve $CD4^+$ T cells to T-helper cells is guided by specific costimulatory cytokines, which mediate the activation of lineage-specific transcription factors and induce the secretion of T cell-specific effector cytokines[42]. As shown in figure 2, TGF-b is needed in the differentiation of both, $T_{reg}$ cells and Th17 cells, whereas Th17 cells further require the presence of IL-6 and IL-23. The differentiation of Th2 cells is driven by IL-4 and differentiation of Th1 cells mainly depends on IFN-y and IL-12. The specific effector cytokines of the different T cell subsets determine the cell associated immunologic function regarding either pro-inflammatory or anti-inflammatory characteristics.

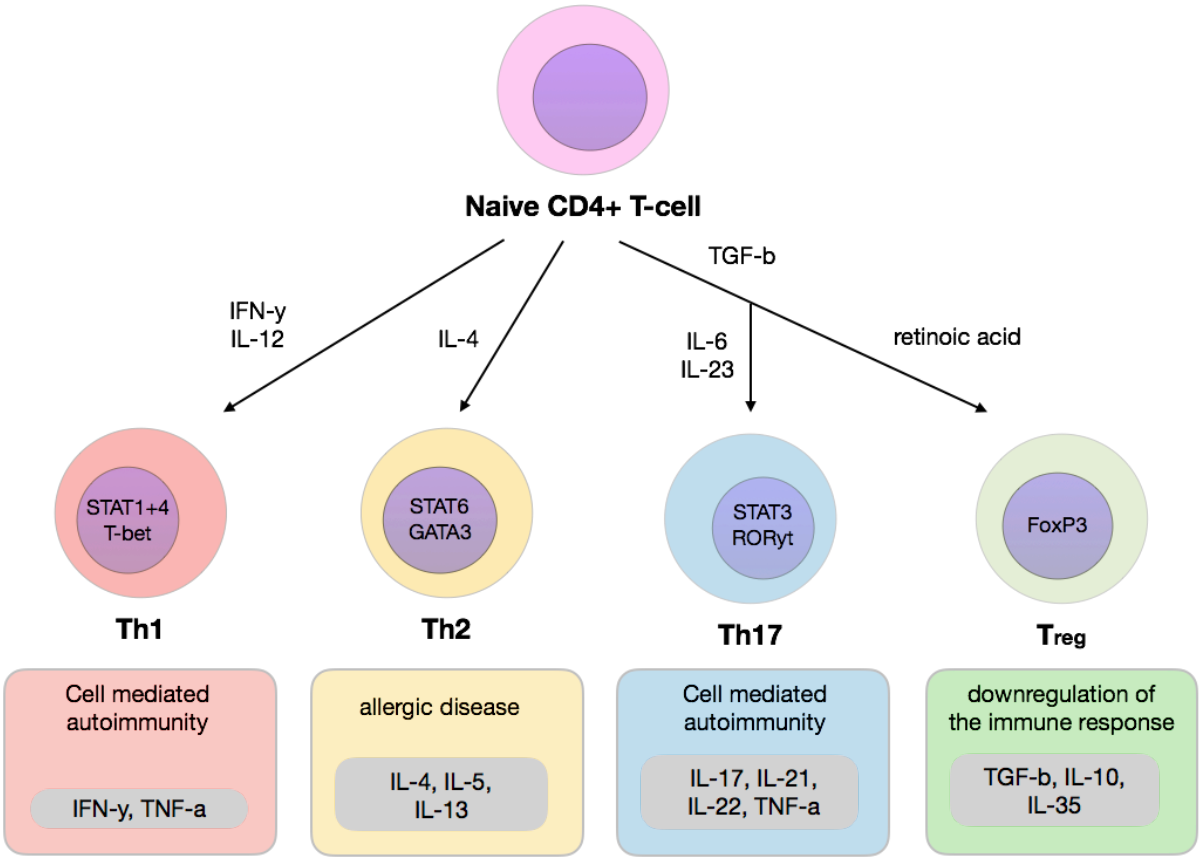


**Figure 2: Differentiation of CD4⁺ T cell subsets** Naïve CD4⁺ T cells differentiate into different T cell subpopulations depending on the present cytokine milieu. The T-helper cell subsets are characterized by the secretion of certain effector cytokines and fulfill different immunologic functions. While Th1 cells and Th17 cells are inducer of cell-mediated autoimmunity, Th2 cells and $T_{reg}$ cells downregulate immune response and maintain self-tolerance. (Figure adapted from Turner et al. 2010[43].)

Th1 cells secrete the pro-inflammatory cytokines IFN-γ and TNF-α and are capable to activate macrophages and induce immune response, whereas immune-modulatory Th2 cells secrete anti-inflammatory cytokines IL-4 and IL-5, which are associated with down-regulation of macrophages and suppression of Th1 cells[44]. For a long time, primarily Th1 cells were presumed to be responsible for initiation of inflammatory processes in RA, because elevated Th1 cell numbers were detected in inflamed joints. Further, Th2 cells were shown to be rather rarely present, leading to the assumption, that an impaired Th1/ Th2 balance might be a driver for autoimmunity[45]. But, with the finding that IFN-γ deficiency even exacerbates the disease while Th17 cell deficiency completely inhibits development of arthritis, new focus of attention was set on the investigation of Th17 cells implicating them as crucial driver in the development of RA[46]. As opponents to the proinflammatory Th17 cells, the regulatory T cells ($T_{reg}$ cells) with their immunosuppressive character also gained more and more importance.

### 3.2.3. Th17 cells

A growing number of studies emphasized that especially the Th17 cells play an important role in the pathogenesis of RA[47-51]. As a crucial factor in defense against extracellular pathogens and infections, Th17 cells have a broad pro-inflammatory capacity which is on the one hand essential to protect the organism, but, on the other hand, needs to be strongly controlled to ensure adequate immune response[52]. Their major cytokine IL-17 functions as a bridge between innate and adaptive immune system and operates self-reinforcing via induction of IL-6 and thereby promotes maintenance of the Th17 cell population in a positive feedback loop[53]. To become an established Th17 cell population, naïve T cells require exposure to a number of cytokines including TGF-β, IL-6, IL-23 and IL-21 (figure 3). While IL-6 and IL-21 promote cell differentiation, survival and expansion, IL-23 stimulation is necessary for the stabilization of the pathogenic phenotype[54,55]. Figure 3 further displays the interaction of Th17 cells with monocytes and fibroblast-like synoviocytes (FLS), showing that Th17 cell differentiation can be induced by cell-to-cell contact of fibroblast-like synoviocytes (FLS) via adhesion molecules[56] or by cell-to-cell contact with activated monocytes via CD147[57]. In contrast to the stimulatory effects on Th17 cells, Th2 cell-associated cytokines and their transcription factor GATA3 are reported to have an inhibitory effect on Th17 cell differentiation and expansion[58]. Accordingly, the differentiation and activation of Th17 cells underlies a complex regulatory network.

**The role of Th17 cells in rheumatoid arthritis**

Let's have a closer look on Th17 cells in RA. Th17 cells highly express cytokine IL-17 as well as other inflammatory effector cytokines like TNF-a, IL-22 and IL-21 (figure 3). These cytokines promote an inflammatory milieu and induce activation, differentiation and survival of other immune cells with a subsequent increase of the inflammatory state[59-61]. Th17 cell effector cytokines can further initiate acute-phase reactions, complement activation and chemokine expression[62] with following recruitment of neutrophils, macrophages and lymphocytes to the synovium[63]. Secretion of cytokine IL-17 can additionally induce angiogenesis through activation of VEGF-secreting synovial fibroblasts[64], and initiate the production of autoantibodies through the activation of specific B lymphocytes[65]. Another pathogenic mechanism ascribed to IL-17 is the interaction with osteoclasts, promoting osteoclastogenesis, joint destruction and focal bone erosions[66].

Patients with RA show highly elevated IL-17 expression levels in peripheral blood[67] and in synovial membranes[68]. While the correlation between serum IL-17 and disease activity is contradictorily reported[69,70], the IL-17 expression in synovial membrane has been reported to be a predictive marker for the progression of joint damage in RA[68]. Due to the high pro-inflammatory properties of Th17 cells and due to the IL-17 hypersecretion in patients with RA,

evidence for the impact of dysregulated Th17 cell populations as critical drivers in the development of disease dramatically increased. The fact that IL-17 receptor A is not only expressed at sites of joints, but also in multiple tissues including skin, lung, intestine and hematopoietic tissues[42] might be interesting in the understanding of extra-articular symptoms and systemic complications.

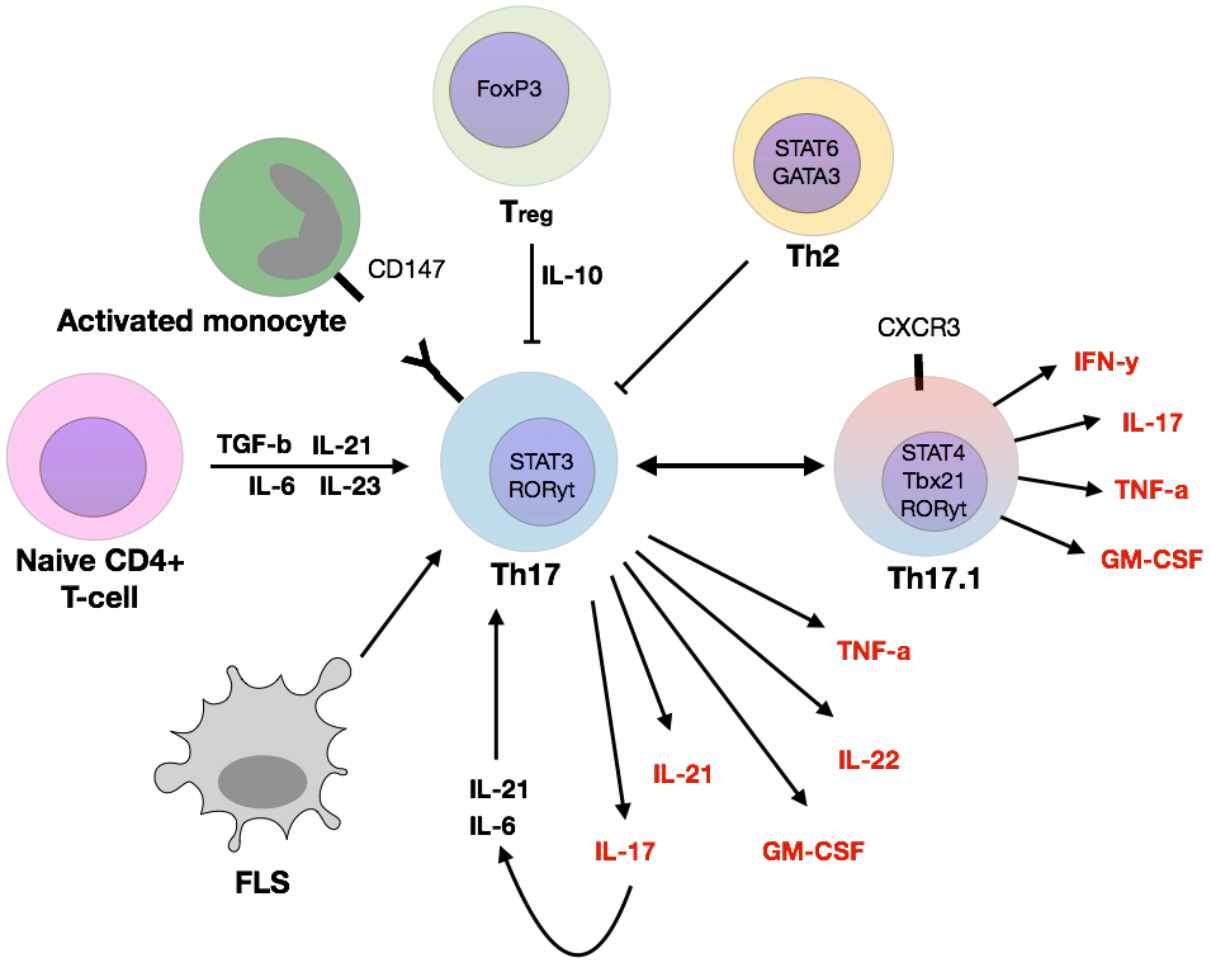


**Figure 3: Th17 cell function.** Th17 cell differentiation is mainly induces by cytokines IL-21, IL-23, IL-6 and TGF-b and by direct cell-to-cell-contact with fibroblast-like synoviocytes (FLS) and activated monocytes. Th17 cells secrete a range of pro-inflammatory cytokines including IL-17, IL-21, IL-22 and TNF-α. The phenotype Th17.1 has its origin in classical Th17 cells and co-produces the Th1 cell related cytokine IFN-γ. Through the secretion of pro-inflammatory cytokines, Th17 cells induce immune response and defend the organism from extracellular pathogens. This strong pro-inflammatory activity is regulated by $T_{reg}$ cells and Th2 cells.

Further investigations suggest that IL-17 producing T cells can be distinguished in two subpopulations based on gene signature and pathogenic character. One of the two subpopulations is formed by classical Th17 cells, the other one is formed by non-classical Th17.1 cells, also named Th1-like Th17 cells or ex-Th17 cells, which are characterized by the additional expression of Th1 associated molecules such as Interferon γ (IFN-γ), STAT4,

CXCR3, and T-box 21 (Tbx21) while the expression of hydrocarbon receptor (AHR) and the anti-inflammatory molecule IL10 is decreased[71]. Thus, in comparison to classical Th17 cells, Th17.1 cells show even higher pathogenic properties with increased ability to proliferate and to produce pro-inflammatory cytokines GM-CSF and TNF. They have further been reported to be more resistant to $T_{reg}$ suppression than classical Th17 cells are[72]. The number of Th17.1 cells is increased in both, synovial fluid at site of inflammation and peripheral blood of patients with RA[73]. The conversion from classical Th17 cells to Th17.1 cells is facilitated by the high instability of the Th17 cell phenotype and occurs under standard inflammatory conditions, being promoted by pro-inflammatory cytokines[74,75].

Thus, there is strong evidence that chronic inflammation and severe pathologic conditions are caused by an upregulated Th17 cell response and high release of Th17 cell-associated pro-inflammatory cytokines. But still, considering the high Th17 cell activation, there might not only be an exceeded stimulation, but also a dysfunction of counteracting immunomodulatory cells that technically act in a suppressive manner in order to regulator immune response. Main representatives of these immunomodulatory cells are the previously mentioned $T_{reg}$ cells.

### 3.2.4. Regulatory T cells and their immunologic function

Regulatory T-cells form a subset of $CD4^+$ T lymphocytes and play a protective role in RA and other autoimmune diseases[76]. Their major function is to maintain self-tolerance and inhibit exceeding (auto)immune response through immunosuppressive mechanisms that affect both, cells from the innate immune system and the adaptive immune system[77]. Depending on their origin, two different $T_{reg}$ subsets were found. While natural $T_{regs}$ ($nT_{regs}$) derive from the thymus by T-cell-Receptor (TCR) stimulation and CD28 co-stimulation, the development of inducible $T_{regs}$ ($iT_{regs}$) occurs in the periphery from naïve T cells and requires the presence of IL-2 and transforming growth factor (TGF)-β[78]. Both subsets are characterized by the expression of transcription factor FoxP3, that is induced by STAT5. But, because FoxP3 is not specific for human $T_{reg}$ cells but can also be expressed in activated T effector cells[79], additional molecules have been applied to characterize $T_{reg}$ cells leading to the common definition as $CD4^+$ $CD25^{hi}$ $CD127^{low}$ cells or $CD4^+$ $CD25^{hi}$ $FoxP3^+$ cells.

The importance of regulatory T cells for the prevention of autoimmunity was discovered by experimental mice model studies with $T_{reg}$ cell depleted collagen induced arthritis (CIA) mice, which are used as the standard animal model of RA. After depletion of $CD4^+CD25^+$ regulatory T cells, more severe inflammation and rapid onset of disease were detected compared to control CIA mice, underlining the profound immunosuppressive function of these cells[80]. Analyzing the mode of action of $T_{reg}$ cells, main suppressive mechanisms were ascribed to the secretion of immunosuppressive cytokines, functional modification of dendritic cells and direct suppression via cell-cell contact[81]. As shown in figure 4, the $T_{reg}$ cell-associated effector

cytokines IL-10, TGF-β and IL-35 are able to inhibit effector functions of autoreactive T cells via receptor binding and thereby reduce cell proliferation and release of pro-inflammatory cytokines[82]. Due to its breaking effect on Th17 cells, particularly IL-10 seems to be essential for the suppressive function of Treg cells[83,84]. Further, regulatory T-cells are able to control cytolysis and regulation of dendritic cell maturation and interact directly with these cells via the membrane-bound molecule cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)[85,86]. CTLA-4 binds to APC surface molecules CD80/CD86 with high affinity and thereby constrains T-cell activation and limits pathologic immune response[87]. Further, a recent mice study revealed that both, $nT_{regs}$ and $iT_{regs}$ directly suppress B cell response by inhibiting cell proliferation and reducing autoantibody production[88]. A crucial role for effective $T_{reg}$ cell function plays the transcription factor FoxP3, which is on the one hand responsible for the early development of $T_{regs}$ in the thymus and, on the other hand, is needed to maintain the suppressive activity of mature peripheral $T_{regs}$[89].

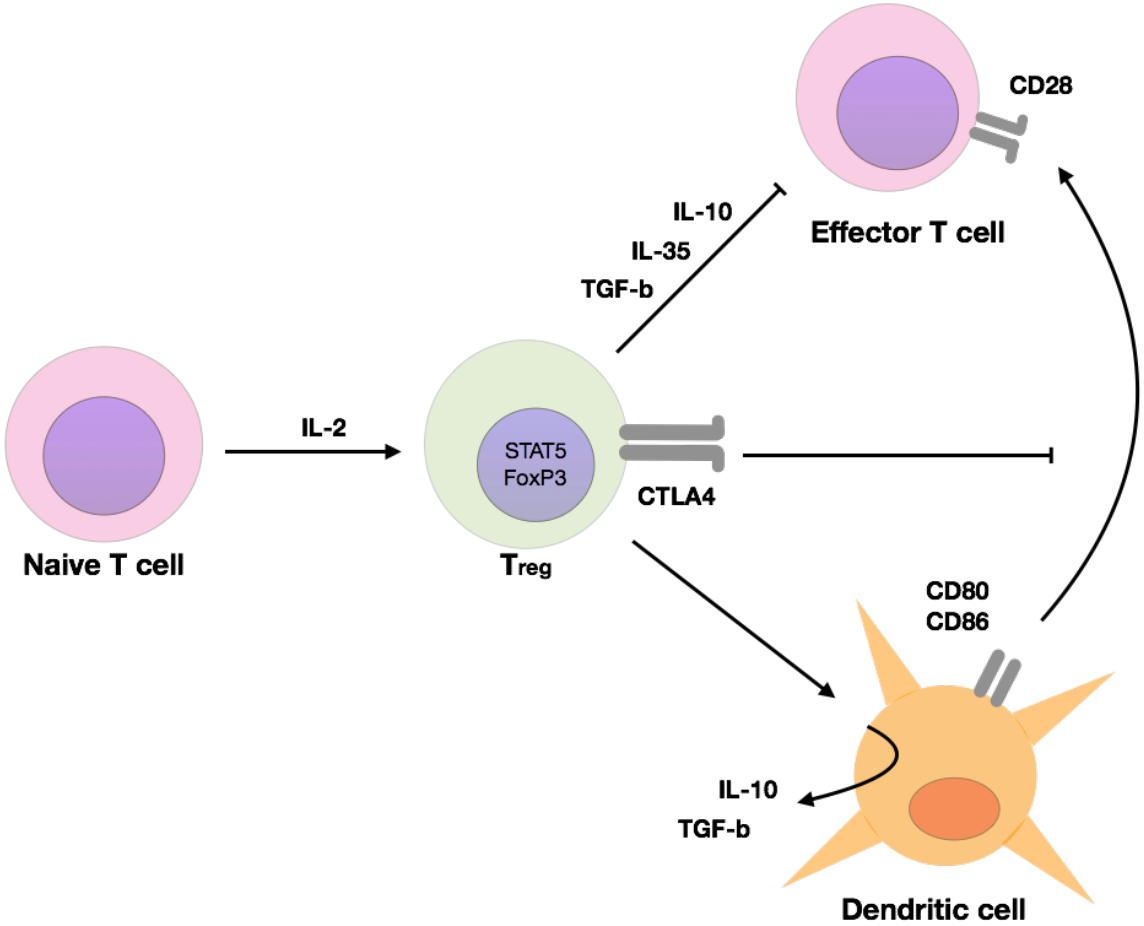


**Figure 4**: **Regulatory T cells: Mechanisms of suppression.** With the secretion of anti-inflammatory cytokines IL-10, IL-35 and TGF-β, regulatory T cells directly inhibit effector functions of autoreactive T cells and contribute to maintenance of self-tolerance. Regulatory T cells also limit T effector cell activation through interaction with dendritic cells, binding their surface molecules CD80/CD86 via CTLA4.

Regarding direct cell-cell interaction between $T_{regs}$ and Th17 cells, it was hypothesized that FoxP3 directly inhibits Th17 cell differentiation through antagonizing the activity of the Th17 specific transcription factor RORC[90] and thereby attenuates Th17 cell functions. Further, IL-2 induced activation of STAT5 seems to play an important role in the $T_{reg}$ cell mediated regulation of Th17 cells, regarding that *in vitro* analyses showed IL-2 dependent decreases of Th17 cells, whereas blocking of IL-2 led to increased Th17 cells and decreased percentage of $FoxP3^+$ cells[91]. Hence, it is evident that immunosuppressive $T_{reg}$ cell function is essential for immune homeostasis and adequately controlled immune responses.

**$T_{reg}$ cell dysfunction in rheumatoid arthritis**

In rheumatoid arthritis, $T_{reg}$ cells were shown to be impaired resulting in uncontrolled proliferation of effector T cells and promotion of inflammatory processes and autoimmunity[92]. Focusing on the causes for $T_{reg}$ cell deficiency, so far, three different mechanisms are taken into account. First, inadequate numbers of $T_{reg}$ cells, second, defective $T_{reg}$ cell function, and third, resistance of effector T cells to $T_{reg}$ cell mediated suppression[93].

Interestingly, the number of regulatory T cells in synovial fluid and peripheral blood of patients with RA was controversially discussed. While van Amelsfort and Han showed an increased frequency of $T_{reg}$ cells[94,95], also unchanged numbers were reported[96,97] and finally, a range of other studies observed decreased $T_{reg}$ cell numbers in patients with RA [98-102] supporting the hypothesis that $T_{reg}$ cell depletion is one reason for reduced immunosuppressive capacity with subsequent inflammatory conditions. Indications for $T_{reg}$ cell dysfunction were primarily revealed by Ehrenstein et al. highlighting the inability of $T_{reg}$ cells isolated from RA patients to suppress cytokine secretion of effector T cells[103]. This defective immunosuppressive function was related to defects in CTLA4 mediated inhibition of T cell receptor signaling caused by reduced CTLA4 expression in RA patients[104].

Furthermore, the inflammatory microenvironment seems to be involved in $T_{reg}$ cell dysfunction. Elevated levels of pro-inflammatory cytokines, especially IL-6, were shown to suppress the expression of FoxP3 and thereby break stability and proliferation of $T_{reg}$ cells[105]. The presence of IL-6 and IL-1β appears to promote plasticity in $T_{reg}$ cells inducing $T_{reg}$ cell conversion towards exFoxp3 Th17 cells with high inflammatory and osteoclastogenic properties and a subsequent breaking of the immunologic balance in favor of proinflammatory Th17 cells[106,107].

Furthermore, a $T_{reg}$ cell resistant phenotype, which enables effector T cells to overcome $T_{reg}$ cell regulated suppression, was described by Haufe et al. They performed *in vitro* proliferation assays with cocultured T effector cells and $T_{reg}$ cells and detected reduced $T_{reg}$ cell suppressive capacity towards CD69 and HLA-DR expressing T effector cells[108]. The resistant phenotype further seems to be based on the one hand, on the extracellular cytokine milieu itself and, on

the other hand, on intracellular signaling molecules like SHP-1, TRAF6 and Cbl-b, that are involved in hyper-activation of the PI3K/Akt pathway[109]. Increased PI3K/Akt signaling was associated with resistant T effector cells, while inhibition of PI3K/Abt restored T effector cell sensitivity to $T_{reg}$ cells[110].

All findings on the functioning of Th 17 cells and $T_{reg}$ cells point out the strong involvement of these two T cell subsets in immunopathogenic processes of RA and other autoimmune diseases. Thus, the Th17/$T_{reg}$ ratio gained focus in the analyses of RA patients and was shown to be critically disturbed compared to healthy controls[111]. Restoring the immunologic imbalance in order to reduce chronic inflammation rapidly became an issue in the treatment of RA. But how can the immunologic imbalance be addressed? Primarily, by targeting molecular key mechanisms that are involved in T cell differentiation and T cell survival. These key mechanisms can for example be found in the JAK/STAT signaling pathway which plays a pivotal role in T cell activation and immune response including the activation of Th17 cells and $T_{reg}$ cells.

## 3.3 The JAK/STAT signaling pathway

The janus kinase (JAK)/ signal transducers and activators of transcription (STAT) signaling pathway plays a crucial role in the differentiation and activation of different T cell subsets. It was detected to be essential for transferring signals from membrane receptors to the nucleus in order to drive controlled immune response[112]. The janus kinase family includes four different enzymes named JAK1, JAK2, JAK3 and TYK2, which are intracellular non-receptor tyrosin kinases located at sites of cytokine receptors binding their intracellular domain. The kinase regions JH4 to JH7 are positive regulator regions crucial for catalytic activity, auto-phosphorylation and interaction with other kinases while the kinase inhibitory region interacts with suppressors of cytokine signaling proteins (SOCS) that mediate negative regulation[113]. The intracellular JAK signaling cascade is induced through cytokine binding to the membrane receptor resulting in conformational changes and auto-phosphorylation of the janus kinases. Once phosphorylated, janus kinases generate docking sites for STATs and promote STAT activation, which enables them to form dimers and translocate into the nucleus[114]. Via binding to specific DNA sequences, STATs regulate gene transcription and expression of different cytokines that are involved in immune response and inflammation[115]. Thus, STAT signaling plays an important role in activating as well as suppressing immune cell development and differentiation influencing the balance between T effector cells and regulatory T cells.

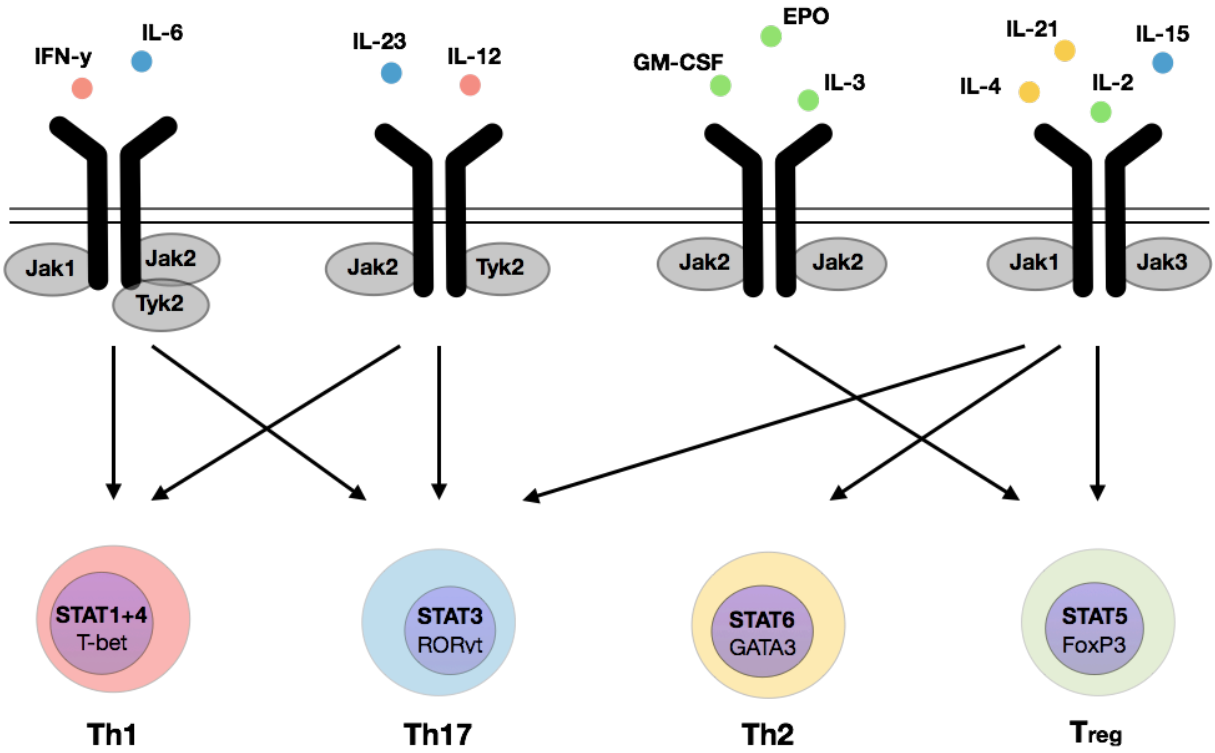


**Figure 5: JAK/STAT signaling.** The janus kinases Jak1, Jak2, Jak3 and Tyk2 are associated to four main cytokine receptor families. Cytokine binding and receptor stimulation leads to activation of the janus kinases. Via downstream STAT phosphorylation, the janus kinases regulate gene transcription and induce the differentiation of distinct T-cell subsets including Th1-cells, Th17-cells, Th2-cells and regulatory T-cells.

While STAT1 and STAT4 activation mainly induces IFN-γ production and Th1 cell differentiation, STAT3 is required for Th17 cell development and IL-17 secretion. Regulatory T cells are induced by STAT5 mediated activation of FoxP3 and Th2 cell differentiation occurs via STAT6 and GATA3[116]. Interestingly, JAK2 is activated by a huge range of ligands and can combine with JAK1, Tyk2 or JAK2 which enables this kinase to form part of most JAK/STAT signaling pathways and to induce not only a single STAT, but STAT1, STAT3, STAT4 and STAT5. Thereby JAK2 is for example involved in IL-6 and IL-23 mediated Th17 cell differentiation through STAT3, but also in IFN-γ/STAT1 mediated Th1 cell polarization as well as $T_{reg}$ differentiation through STAT5 [117]. The complexity of JAK/STAT signaling has further been underlined by reciprocal regulatory mechanisms between Th17 cells and $T_{reg}$ cells regarding that IL-6 downregulates FoxP3, while FoxP3 was shown to inhibit Th17 cell differentiation via antagonizing the transcription factor RORγt [90].

In RA, reciprocal control and regulatory mechanisms for adequate immune responses seem to be defective. It was shown that expression of JAKs and STATs in rheumatoid arthritis differs

from healthy individuals and might play a role in the pathogenesis of RA[118]. Walker et al. showed increased levels of JAK3, STAT4 and STAT6 in synovial tissue of RA patients and correlated them to the presence of serum rheumatoid factor implicating the components of the JAK/STAT pathway as potential targets for the treatment of RA[119]. Especially JAK3 was focused regarding the development of potential treatment targets due to the fact that JAK3 is only present on lymphocytes and dendritic cells, whereas all other janus kinases are ubiquitously expressed. Further, JAK3 deficiency was reported to result in severe combined immunodeficiency (SCID) with serious life-threatening infections, so that JAK3 was supposed to be main driver in inflammatory responses and autoimmunity[120].Thus, targeting janus kinases for the treatment of RA appeared to be promising. Additionally, when taking into account that the inhibition of IL-6 mediated JAK/STAT signaling by administration of IL-6R blocker tocilizumab already showed significant efficiency in the decrease of Th17 cells and increase of regulatory T cells[121].

### 3.3.1. JAK inhibitors for the treatment of rheumatoid arthritis

Considering the pivotal involvement of the JAK/STAT pathway in differentiation and activation of T cells, JAK inhibitors were developed with the aim to inhibit downstream activation of pathogenic effector T cells especially Th17 cells. JAK inhibitors were supposed to diminish their proliferation and thereby attenuate immunity and reduce disease activity. After proving efficacy in the treatment of other autoimmune diseases, JAK inhibitors recently also achieved approval for the treatment of rheumatoid arthritis. First JAK inhibitor in clinical use for RA treatment was tofacitinib, a pan-JAK inhibitor targeting mainly JAK3 and JAK1, but also with smaller effect JAK2 and TYK2[122]. Successfully passing clinical trials tofacitinib was granted European Medicines Agency (EMA) approval in 2017[123]. At the same time, the JAK1 and JAK2 inhibitor baricitinib was approved showing significant clinical improvement and efficacy in several clinical phase 3 trials of RA patients[124-127]. Regarding the safety profile of JAK inhibitors, adverse events like serious infections, malignancies and hematological abnormalities were largely reported in an acceptable number similar to conventional synthetic disease modifying antirheumatic drugs (csDMARDs) like methotrexate (MTX), sulphasalazine and leflunomide, to biological (b)DMARDs like TNFα inhibitors and similar to other antibody treatments like abatacept and tocilizumab[128]. A very recent randomized non-inferiority study by Ytterberg et al. firstly observed a higher risk of major adverse cardiovascular events (MACE) and cancer in tofacitinib treated patients compared to TNF inhibitor treated patients in a four-year follow-up. Their study population was built by a special group of patients with active RA despite MTX treatment at an age of 50 years or older and with at least one cardiovascular risk factor[129]. Hence, the increased risk of MACE and cancer is not generally transferable to all RA patients but should be kept in mind when starting a new therapy with tofacitinib. JAK inhibitors are

classified as targeted synthetic (ts)DMARDs and showed great advantages in clinical use due to their oral application in contrast to other DMARDs which were subcutaneously or intravenously applicated. Additionally, it occurred that treatment with biological DMARDs loosed efficacy caused by the development of antidrug antibodies[130], which was so far not detected after treatment with JAK inhibitors. Hence, accounting drug efficacy, safety, side effect profile costs and the still missing information of long-term studies, JAK inhibitors were finally recommended for the treatment of MTX refractory RA with an equal value as biological DMARDs[131].

Even though JAK inhibitors have proven efficacy in several clinical studies, the detailed molecular effects on T cell differentiation, cytokine secretion and immune signaling in patients with RA are only gradually discovered. Doing research on the JAK/STAT signaling pathway and on the effect of JAK inhibitors on T cell functions provides great potential in extending our understanding of immunopathogenesis. Based on new findings on immune signaling in rheumatoid arthritis, treatment strategies and disease management will be further improved.

## 3.4 Purpose of the study

Bringing together the new approval of JAK inhibitors for treatment of rheumatoid arthritis and the pivotal role of Th17 cells and regulatory T cells in the pathogenesis of autoimmunity, this study was constituted to investigate the molecular effect of the JAK inhibitors baricitinib and tofacitinib on JAK/STAT mediated activation and differentiation of Th17 cells, regulatory T cells and the Th17/$T_{reg}$ balance. On the one hand, we questioned the ability of JAK inhibitors to restore the impaired Th17/$T_{reg}$ balance and thereby especially focused on their effect on regulatory T cells, which has not been described before. On the other hand, we aimed to reveal potential differences in the molecular effect of the two different JAK inhibitors baricitinib and tofacitinib considering that they target different janus kinases.

# 4. Material and methods

## 4.1 Study population

Peripheral whole blood samples were obtained from patients with established rheumatoid arthritis and healthy controls by the Clinic for Internal Medicine I at the University Hospital of Cologne. Blood samples were taken in a defined volume of 15-17ml in two EDTA S-monovettes and were processed within 24h.

An overall number of 63 patients with established rheumatoid arthritis, all fulfilling the ACR/EULAR criteria for the classification of RA, were included into the study. Depending on the treatment, RA patients were sub-classified in tofacitinib-treated, baricitinib-treated, MTX-treated and untreated RA patients. Treated patients were included when receiving single drug treatment for minimum four weeks. Untreated patients were defined as active RA patients without treatment for at least four weeks. The group of healthy controls was formed by 15 patients from the immunologic outpatient clinic without autoimmune disease and without receiving any immune-modulatory treatment. General exclusion criteria of the study were acute infections at the time of blood draw and proof or suspicion of additional chronic inflammatory diseases.

All patients included in the study were of full age and provided written informed consent of participation. In order to fulfill data privacy, all patients immediately received a consecutive study number.

## 4.2 Material

*Buffer*

| | |
|---|---|
| Hanks salt Solution 1x Phosphate Buffered Saline (PBS) | BioChrome AG Berlin, Germany |
| autoMACSpro Running Buffer | Miltenyi Biotec GmbH, Bergisch Gladbach, Germany |
| autoMACSpro Washing Buffer | Miltenyi Biotec GmbH, Bergisch Gladbach, Germany |

*Medium*

| | |
|---|---|
| X-VIVO™ 15 | Lonza, Verviers, Belgium |
| Human Serum Albumin | Sigma-Aldrich, Saint Louis, USA |
| Penicillin-Streptomycin | Sigma-Aldrich, Saint Louis, USA |

*Reagents*

| | |
|---|---|
| Ionomycin, Calcium Salt | Cell Signaling Technology, Denver, USA |
| Phorbol Myristate Acetat (PMA) | Cell Signaling Technology, Denver, USA |
| Brefeldin A (1000x Solution) | eBioscience, San Diego, USA |
| Pancoll | PAN TM Biotech GmbH, Aidenbach, Germany |
| TaqMan Fast Advanced Master Mix | Applied Biosystems, ThermoFisher Scientific, Carlsbad, U.S. |
| RNAse free water | Qiagen, Hilden, Germany |
| FlowClean Cleaning Agent, 500ml | Beckman Coulter, Krefeld, Germany |
| Trypan blue stain 0,4% | Life Technologies Corporation, Carlsbad, U.S. |

*Antibodies*

| **Name** | **Epitope** | **Fluorophore** | **Isotype** | **Species** | **Clone** | **Company** | **Lot** |
|---|---|---|---|---|---|---|---|
| Pacific Blue™ anti-human CD4 Antibody | CD4 | Pacific Blue | Mouse IgG2b,κ | Mouse | OKT4 | BioLegend® | B237660 |
| IL-17A Monoclonal Antibody | IL-17A | PerCP-Cy5.5, eFluor 660 | Mouse IgG1, κ | Mouse | eBio64DEC17 | eBioscience™ | 4336371 |
| FITC anti-human IFN-γ Antibody | IFN-γ | FITC | Mouse IgG1, κ | Mouse | | BioLegend® | |
| PE anti-human CD25 Antibody | CD25 | PE | Mouse IgG1, κ | Mouse | M-A251 | BioLegend® | B211745 |
| FITC anti-human CD127 (IL-7Rα) Antibody | CD127 | FITC | Mouse IgG1, κ | Mouse | A019D5 | BioLegend® | B242093 |

**Table 2: Characteristics of used antibodies.**

*PCR-primer*

| **Target** | **Primer Nr.** | **Lot** | **Company** |
|---|---|---|---|
| β-2-microglobulin (B2M) | Hs00984230_m1 B2M | 1693102 | Applied Biosystems, ThermoFischer, Carlsbad, U.S |

| | | | |
|---|---|---|---|
| Retinoic-acid-related-orphan receptor (RORC) | Hs01076122_m1 RORC | 1469511 | Applied Biosystems, ThermoFischer, Carlsbad, U.S |
| T-box 21 (TBX21) | Hs00203436_m1 TBX21 | 1194419 | Applied Biosystems, ThermoFischer, Carlsbad, U.S |
| Forkhead-box-protein P3 (Foxp3) | Hs01085834_m1 FoxP3 | 1133662 | Applied Biosystems, ThermoFischer, Carlsbad, U.S |
| Janus tyrosine kinase 1 (Jak1) | Hs01026983_m1 JAK1 | 1679476 | Applied Biosystems, ThermoFischer, Carlsbad, U.S |
| Janus tyrosine kinase 2 (Jak2) | Hs01078136_m1 JAK2 | 1669760 | Applied Biosystems, ThermoFischer, Carlsbad, U.S |
| Janus tyrosine kinase 3 (Jak3) | Hs00354555_m1 JAK3 | 1669760 | Applied Biosystems, ThermoFischer, Carlsbad, U.S |
| Tyrosine kinase 2 (Tyk2) | Hs01105947_g1 TYK2 | 1645052 | Applied Biosystems, ThermoFischer, Carlsbad, U.S |

**Table 3: Primers used for PCR analysis.**

*Kits*

| | |
|---|---|
| $CD4^+$ T Cell isolation Kit | Miltenyi Biotec GmbH, Bergisch Gladbach, Germany |
| BD Cytofix/Cytoperm TM Fixation/Permeabilization | BD Bioscience, Heidelberg, Germany |
| RNeasy Mini Kit | Qiagen, Hilden, Germany |
| Quantitect Reverse Transcription Kit | Qiagen, Hilden, Germany |

*Equipment*

| | |
|---|---|
| Megafuge 1.OR | Heraeus Insturments Düsseldorf, Germany |
| AutoMACS Pro Separator | Miltenyi Biotec GmbH, Bergisch Gladbach, Germany |
| $CO_2$ Incubator | Binder GmbH, New York, USA |
| 7500 Fast Real-Time PCR System | Applied Biosystems, ThermoFisher Scientific, Carlsbad, U.S. |
| ZX3 Advanced Vortex Mixer | VELP Scientifica, Usmate Velate MB, Italy |
| Mars Safety class 2 ScanLaf | Labogene, Denmark |
| Gallios TM flow cytometer | Beckman Coulter, Krefeld, Germany |
| Countess II FL Automated Cell Counter | Applied Biosystems, ThermoFisher Scientific, Carlsbad, U.S. |

| | |
|---|---|
| Nanodrop 1000 | ThermoFisher Scientific, Carlsbad, U.S |
| ThermoStat TM 5320 | Eppendorf, Wesseling, Germany |
| Freezer -80°C | Panasonic, Germany & Austria |
| Freezer -20°C | Liebherr Premium, Bulle FR, Switzerland |
| Fridge 4°C | Liebherr Premium, Bulle FR, Switzerland |

*Software*

| | |
|---|---|
| Kaluza | Kaluza Software 2.1 |
| Graph Pad Prism | Graph Pad Software |
| Adobe illustrator CC | Adobe |

## 4.3 Methods

All methods occurred under S1 safety conditions in a sterile hood Mars Safety class 2.

### 4.3.1. Cell isolation

*Density-gradient centrifugation*

Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll® Hypaque separation medium. Therefore, whole blood samples were diluted 1:1 with Phosphate Buffered Saline (PBS) and gently provided above Ficoll® Hypaque solution in a 50ml falcon tube. The blood samples were then centrifuged for 20min at 1600rpm at room temperature without break in order to divide blood components according to their sedimentation rate. The bottom layer was formed by cells with high density such as erythrocytes and dead cells. The white interphase contained the PBMCs and the upper layer was formed by blood serum and thrombocytes (figure 6). PBMCs were collected in a 15ml falcon tube and washed with PBS, following a centrifugation of 10min at 1600rpm at room temperature.

*$CD4^+$ T cell isolation*

$CD4^+$ T cells were extracted from PBMCs by magnetic separation using a $CD4^+$ T cell extraction kit (Miltenyi Biotec) and the autoMACS Pro Separator according to the manufacturer's instructions. The purity was confirmed by $CD4^+$ fluorescence-activated cell sorting (FACS) analyses.

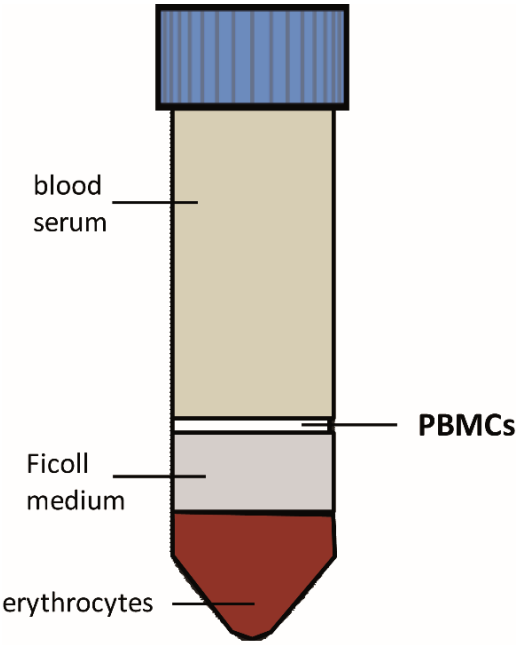


**Figure 6**: **Blood layers after density-gradient centrifugation.** Erythrocytes assemble at the bottom and are covered by a Ficoll medium layer. PBMCs form a small interphase between Ficoll medium and blood serum.

*Cell number identification*

The number of isolated $CD4^+$ T cells was analyzed by Countess II FL Automated Cell Counter. Therefore $CD4^+$ T cells were suspended in 1ml X-Vivo medium (X-VIVO$^{TM}$ 15, 1% human serum, 1% penicillin-streptomycin) and 10µl of the cell suspension were diluted 1:1 with trypan blue to be then transferred to a Cell Countess counting chamber. Trypan blue is not absorbed by vital cells due to their intact cell membrane, while dead cells absorb the colorant. Thereby the cell counter machine is able to distinguish dead and vital cells and display the number of vital cells calculated per a volume of 1ml.

### 4.3.2. Flow cytometry

Flow cytometry is a method used to characterize cell populations by detecting morphologic and immunologic properties such as size, granularity, membrane-bound or intracellular molecules and receptors. Cell characteristics are detected by a laser beam generating a cell specific light scatter while cells flow through. The forward scatter shows cell size, while the side scatter detects cell granularity. In order to quantitatively determinate membrane-bound and intracellular molecules, cells are marked with fluorochrome-conjugated antibodies and further fluorescence intensity of emitted light is measured. A high intensity implies a high number of antibody- bound cells.

*Antibody stainings*

Extracellular staining: After isolation, $CD4^+$ T cells were stained extracellularly with anti-CD4 antibody, anti-CD25 antibody and anti-CD127 antibody to show regulatory T cells. Therefore $CD4^+$ T cells were transferred to conical round-bottom tubes, then washed with 1ml PBS and centrifuged at 1500rpm for 3min. Supernatant has been removed and cells were incubated with the certain fluorochrome-conjugated antibodies (table 2), all in a volume of 1µl per 1x10^6 cells. After incubation for 20min at 4°C in the dark, cells were washed with 1ml PBS, centrifuged again at 1500rpm for 3min and re-suspended in 500µl PBS to proceed to flow cytometry.

Intracellular staining: Peripheral blood isolated CD4+ T cells were plated at 1x10^6 cells in 800µl X-Vivo medium and stimulated with 2,4µl Ionomycin (1 mM) and 8µl Phorbol 12-Myristate 13-Acetate (PMA, 10ng/ml) to induce the secretion of intracellular cytokines. After 1h of stimulation, Brefeldin A (BFA), a lactone antiviral and protein transport inhibitor, was added for 3-4h. The stimulation occurred under standard terms (37°C, 5% CO2) in a 24-well plate for cell suspension. After a total stimulation time of 4-5h, CD4+ T cells were transferred to conical round-bottom tubes, washed with 1ml PBS and centrifuged at 1500rpm for 3min. Supernatant has been removed and cells were fixed and permeabilized with BD Cytofix/Cytoperm TM Fixation/ Permeabilization Kit by adding 100µl of the BD Cytofix/Cytoperm solution to each tube. Cells were briefly mixed and incubated for 20min at 4°C in the dark. Fixed and permeabilized cells were washed with 1 ml BD Perm/Wash TM Buffer (diluted 1:10 with distilled H2O) and then incubated with intracellular fluorochrome-conjugated antibodies IL-17A PerCP Cy5.5 and IFNy FITC at a volume of 2µl per 1x10^6 cells for 20min at 4°C in the dark. After antibody staining, cells were again washed with 1ml BD Perm/Wash buffer, centrifuged at 1500rpm for 3min, and finally re-suspended in 500µl BD Perm/Wash buffer to proceed to flow cytometry.

*FACS analysis*

Cytokine concentrations and membrane-bound molecules were measured with Gallios TM Flow Cytometer. For each measurement an acquisition of 20.000 to 25.000 cells was performed. LMD data from fluorescence activated cell sorting (FACS) measurement was analyzed by Kaluza software (Analysis version 2.1).

### 4.3.3. PCR

In order to analyze the effect of janus kinase inhibitors at gene expression level, cells were analyzed by Reverse Transcriptase - quantitative Polymerase Chain Reaction (RT-qPCR) and

janus kinase expression of *JAK1, JAK2, JAK3* and *TYK2* as well as cell specific transcription factors *RORC, TBX21* and *FOXP3* were quantitatively assessed as described below.

*Ribonucleic acid (RNA) isolation*

Ribonucleic acid was isolated from $CD4^+$ T cells using the RNeasy Mini Kit (Qiagen). Following the manufacturer's instructions, cells were lysed, homogenized and suspended in ethanol to provide ideal binding conditions. Lysate was then loaded onto RNeasy silica membrane and contaminants were washed away. Concentrated RNA was eluted with RNase free water and spectrophotometrically measured by Nanodrop 1000 (ThermoFisher Scientific) at a wavelength of 260nm.

*cDNA synthesis*

Complementary DNA was synthesized from 1000ng RNA using QuantiTect Reverse Transcription Kit (Qiagen). According to the instructions, purified template RNA was first briefly incubated in gDNA Wipeout Buffer to remove genomic DNA, and then incubated with Reverse Transcription MasterMix in order to transcribe the RNA into cDNA. The Reverse Transcription MasterMix contained Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT Primer Mix.

*TaqMan polymerase chain reaction*

TaqMan polymerase chain reaction is a method to measure the amplification of specific nucleic acid sequences via detecting fluorescence signals in real-time. The mechanism is based on so called TaqMan probes, with a fluorescent reporter at one end and a quencher at the opposite end, that anneal to the DNA region. The quencher quenches the fluorescence via Förster resonance energy transfer (FRET) as long as reporter and quencher are in proximity. Once the Taq polymerase synthesized nascent strand and degraded the TaqMan probe with its exonuclease-activity, the reporter-quencher proximity is broken. Fluorophore release consequently allows unquenched fluorescence emission that can be detected with a laser. The fluorescence signals directly correlate with the amount of DNA templates and permit exact quantification.

For DNA amplification the RT-qPCR Master Mix was prepared according to the following table (table 4). The oligonucleotidprimer B2M, Jak1, Jak2, Jak3, Tyk2, RORC, Tbx21 and FoxP3 were used as target DNA sequences.

| **Master Mix RT-qPCR** | **vol/well** |
|---|---|
| TaqMan Fast Advanced Master Mix<br>▪ thermostable *AmpliTaq Fast DNA Polymerase*<br>▪ Mg+ ions<br>▪ desoxyribonucleotides (dNTPs) | 10µl |
| RNAse free water | 8µl |
| Oligonucleotidprimer | 1µl |

**Table 4: Preparation scheme for RT-qPCR Master Mix.** The used Master Mix for RT-qPCR procedure contains TaqMan Master Mix, RNase free water, and the particular oligonucleotidprimer, all in certain volumes to ensure a total Master Mix volume of 19µl per well.

The Master Mix was added to a 96-well PCR plate (*MicroAmp R Fast Optical 96-Well Reaction Plate*, Applied Biosystems) and merged with 1µl cDNA up to a total volume of 20µl per well. The reaction run was performed with a 7500 Fast Real Time PCR System (Applied Biosystems) under the following conditions:

Instrument: 7500 Fast (96 wells)

Type: Quantitation - Comparative $C_T$ ($\Delta\Delta C_T$)

Reagents to detect target sequence: TaqMan® Reagents

Speed: Fast

Cycles: holding stage 95°C, 02.00min; cycling stage step 1: 95°C, 00.03min; cycling stage step2 60°C, 00.30min

*Analyses and statistics*

The relative quantities were obtained using the comparative threshold C method and were normalized to B2M (housekeeping gene). All experiments were performed in triplicate.

All data were evaluated with *GraphPad Prism (GraphPad Software)* by analyses of one-way ANOVA in mean with Standard Error of Difference (SED). Correlations were analyzed by bivariate regression with information on $r^2$ as value for the goodness of fit. P values less than 0.05 were considered significant.

# 5. Results

## 5.1 Patients

In our study we analyzed a population of 63 patients with rheumatoid arthritis, separated in 4 groups according to their current treatment. From all RA patients, 10 patients were untreated, 20 were baricitinib treated, 13 were tofacitinib treated and 20 MTX treated. Healthy individuals served as controls.

Characteristics of the study population are displayed in the following table (table 5). From all untreated RA patients, 7 were female and 3 were male. At time of blood draw, their average of age was 70.2 ± 3.32 years, which is significantly higher than the average of age in baricitinib (55.5 ± 2.40) and tofacitinib (52.7 ± 2.67) treated patients and healthy controls (50.1 ± 2.57). Thus, healthy controls and rheumatoid arthritis untreated patients were not age-matched, due to individual patient characteristics. Advanced age in untreated RA might be caused by age-related increase of adverse events with temporary stop of treatments. In all patient groups, the majority of patients were female and baricitinib treated patients were even exclusively female. Regarding the precise female/ male proportion, there were still differences between the analyzed groups. At this point, a gender related impact on the results cannot be excluded. However, according to a multicentric study performed in Italy with 182 patients, there were no differences in clinical response to JAK inhibitor treatment between female and male patients[132].

The highest mean values of the laboratory inflammation parameters c-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were observed in tofacitinib treated patients (CRP 16.36 mg/l ± 5.058; ESR 28.08 mm/h ± 4.593), showing very divergent CRP values with a range from 0.6 mg/l to 53.3 mg/l, while CRP levels in the other patient groups only slightly range inside the group. The mean CPR level is significantly increased in tofacitinib treated patients compared to baricitinib or MTX treatment. Difference in CRP levels was also observed in untreated RA patients compared to healthy controls, showing significantly elevated CRP values in untreated patients. Beside highly increased ESR values in tofacitinib treated patients, all other groups show similar mean ESR values between 13.5 mm/h in baricitinib and 17.53 mm/h in healthy controls. Although ESR is assessed as a marker for disease activity, we could not observe increased ESR levels in patients with untreated RA compared to healthy controls.

The Disease Activity Score 28 (DAS28) was not assessed for every patient due to structural difficulties. Hence, DAS28 was assessed for 9 baricitinib treated patients, 5 tofacitinib treated patients and 8 MTX treated patients. Values $\leq$ 2.6 are considered as clinical remission, ≤3.2 as low disease activity, >5.1 as very active disease and in between as moderate RA. From all assessed DAS28, only one was higher than 5.1, showing very active RA among the MTX treated patients. All other assessed patients were in a state of inactive or moderate disease at time of blood draw. Approximately half of the included patients treated with baricitinib,

tofacitinib and MTX were rheumatoid factor (RF) positive, whereas the percentage of RF positive patients was incidentally higher in untreated RA patients.

| | HC (n=15) | RA untreated (n=10) | RA Baricitinib (n=20) | RA Tofacitinib (n=13) | RA MTX (n=20) |
|---|---|---|---|---|---|
| **age, years (SD)** | 50.1 (2.57) | 70.2 (3.23) | 55.5 (2.4) | 52.7 (2.67) | 64.9 (2.99) |
| **male/female** | 3/12 | 3/7 | 0/20 | 2/11 | 2/18 |
| **CRP mg/l (SD)** | 2.38 (0.39) | 5.300 (1.29) | 2.49 (0.73) | 16.36 (5.06) | 3.68 (0.98) |
| **ESR mm/h (SD)** | 17.53 (3.76) | 15.90 (1.95) | 13.50 (1.77) | 28.08 (4.59) | 16.79 (3.87) |
| **DAS28** | - | - | 3.16 | 4.22 | 3.12 |
| **RF positive %** | - | 88 | 45 | 50 | 50 |

**Table 5: Characteristics of the study population**. DAS28 was only assessed in 9 baricitinib treated patients, 5 tofacitinib treated patients and 8 MTX treated patients. RF (rheumatoid factor), DAS28 (Disease Activity Score 28), CRP (c-reactive protein).

## 5.2 Th17 cells in rheumatoid arthritis

### 5.2.1. Treatment with baricitinib and tofacitinib significantly reduces IL-17 expression

In order to analyze the Th17 cell population, $CD4^+$ T cells were isolated from human peripheral blood and stained with intracellular antibodies anti-IL-17 PerCp Cy5.5 and anti-Interferon γ FITC. IL-17 positive cells were detected by flow cytometry according to the exemplary gating scheme figure 7. All experiments conform to the same gating strategies.

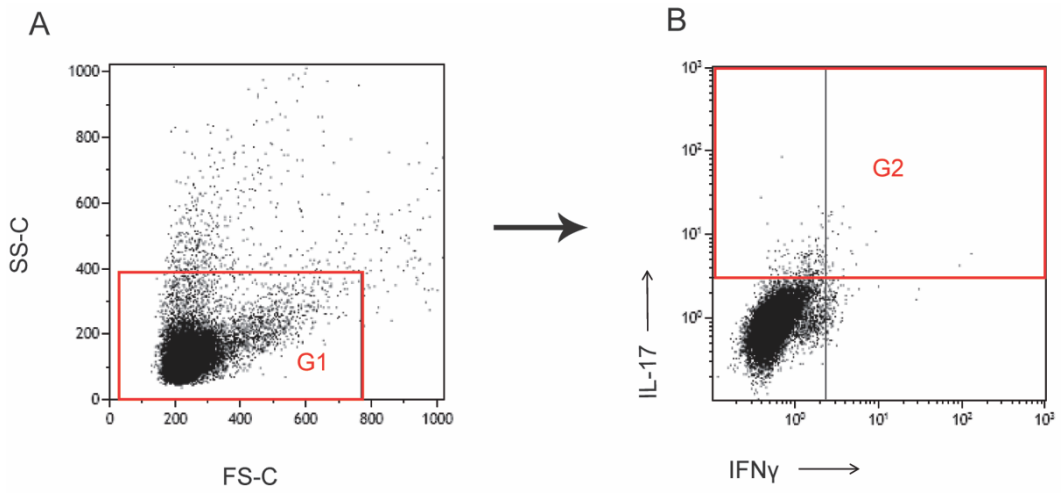


**Figure 7: Detection of Th17 cells by flow cytometry**. **A)** $CD4^+$ T cells in forward-scatter and sideward-scatter. Scattering was constricted with gate G1. **B)** Gating scheme for IL-17

expressing $CD4^+$ T cells defined as Th17 cells. Th17 cells are shown in gate G2. SS-C: sideward-scatter; FS-C: forward-scatter.

IL-17 expression was measured in untreated RA patients, in patients receiving single treatment with baricitinib, tofacitinib or MTX and in healthy controls. As displayed in figure 8, rheumatoid arthritis untreated patients showed the highest frequencies (3.50 % ± 0.63) of IL-17 positive $CD4^+$ T cells (Th17 cells) within the $CD4^+$ T cell population. The percentage of Th17 cells in baricitinib (1.48% ± 0.20), tofacitinib (1.36% ± 0.14) and MTX (1.22% ± 0.19) treated patients was similar to the percentage of Th17 cells in healthy controls (1.31% ± 0.18). Thus, the data show that Th17 cells in patients with RA were significantly diminished by treatment with baricitinib ($p$=0.0006), tofacitinib ($p$=0.0027) and MTX ($p$=0.0003).

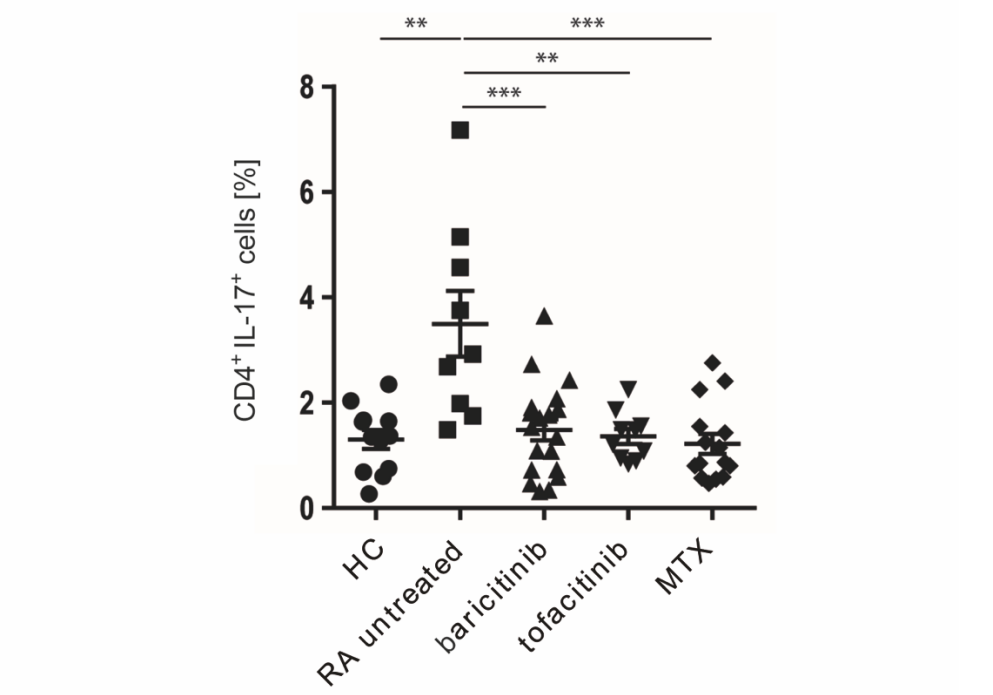


**Figure 8: Significant reduction of Th17 cells by treatment with baricitinib, tofacitinib and MTX.** Percentage of IL-17 positive cells in $CD4^+$ T cells from peripheral blood was analyzed by flow cytometry according to the gating scheme in figure 7. Patients with untreated rheumatoid arthritis (n=9) show highly increased percentage of $IL\text{-}17^+$ $CD4^+$ T cells (Th17 cells). Treatment with baricitinib (n=19), tofacitinib (n=10) and MTX (n=15) significantly reduces the frequency of Th17 cells and readapts it to the mean expression level of healthy controls (n=12). No difference in frequency of Th17 cells was observed between tofacitinib and baricitinib treated patients. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. P-values were calculated using one-way ANOVA. Data are shown as mean ± SEM.

### 5.2.2. IL-17$^+$ IFNy$^+$ double positive Th17 cells in rheumatoid arthritis

The Th17 cells expressing both, IL-17 and IFNy, were analyzed by flow cytometry according to gating scheme figure 9A. For IFNγ staining the intracellular antibody IFN-γ FITC was used. The percentage of IL-17 and IFNy expressing Th17 cells in CD4$^+$ T cells in untreated RA patients was significantly higher (2.06% ± 0.61, $p$=0.0052) compared with healthy controls, which showed a mean percentage of 0.40% ± 0.06. Among all treated patients the average of IL-17 and IFNy positive Th17 cells was very similar (baricitinib 0.54% ± 0.08; tofacitinib 0.44% ± 0.07; MTX 0.40% ± 0.08. Overall, treatment with both JAK inhibitors, baricitinib ($p$= 0.0015) and tofacitinib ($p$=0.0126), as well as treatment with MTX ($p$=0.0021) significantly reduced the percentage of double positive Th17 cells compared to rheumatoid arthritis untreated patients (figure 9B).

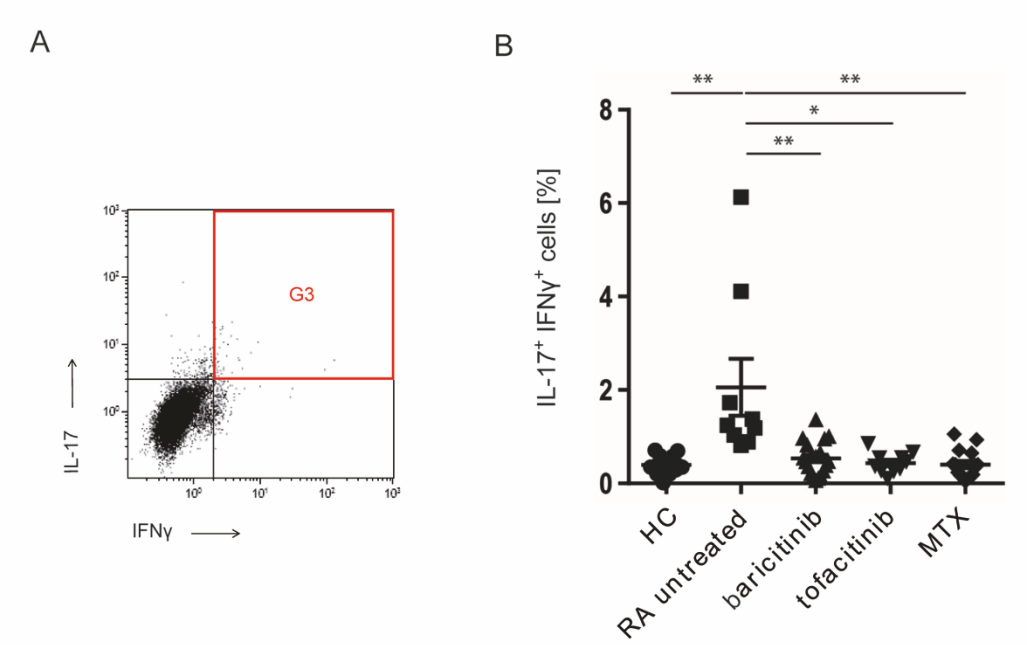


**Figure 9: IL-17$^+$IFNγ$^+$ double positive pathogenic Th17 cells are highly expressed in patients with untreated RA.** Flow cytometry analysis of the percentage of double positive Th17 cells in peripheral blood CD4$^+$ T cells. **A)** Gating scheme for the analyses of IL-17$^+$ IFNγ$^+$ cells. Double positive cells are shown in gate G3. **B)** Percentage of IL-17$^+$ IFNγ$^+$ Th17 cells in CD4$^+$ T cells. Double positive cells are significantly elevated in untreated RA compared to healthy controls. Treatment with baricitinib, tofacitinib and MTX significantly reduces the number of IL-17$^+$ IFNγ$^+$ cells. *$p$ < 0.05, **$p$ < 0.01, *** $p$ < 0.001. P-values were calculated using one-way ANOVA. Data are shown as mean ± SEM.

In addition to the analysis of IFNγ$^+$ Th17 cells within the CD4$^+$ T cells, the percentage of this Th17.1 subpopulation was also analyzed within the Th17 cells to examine at least descriptively potential differences in the Th17 cell plasticity towards high pathogenic Th17.1 cells.

Therefore, IL-17 expressing cells were selected as described before (gate G2), and then shown in a new graph with gating on IFNγ positive cells following gating scheme figure 10A. The percentage of Th17.1 cells within the Th17 cell population in patients with untreated RA showed a mean of 55.83% ± 6.166 and was significantly increased ($p$=0.0116) compared to healthy controls with a mean of 34.98% ± 4.533. Treatment with baricitinib, tofacitinib and MTX significantly reduced their frequency compared to untreated RA patients in a similar extend. The average of the double positive subpopulation within Th17 cells was almost equal among all treatments and is located between 33.73% ± 3.23 in MTX and 36.45% ± 2.55 in baricitinib treated patients (figure 10B).

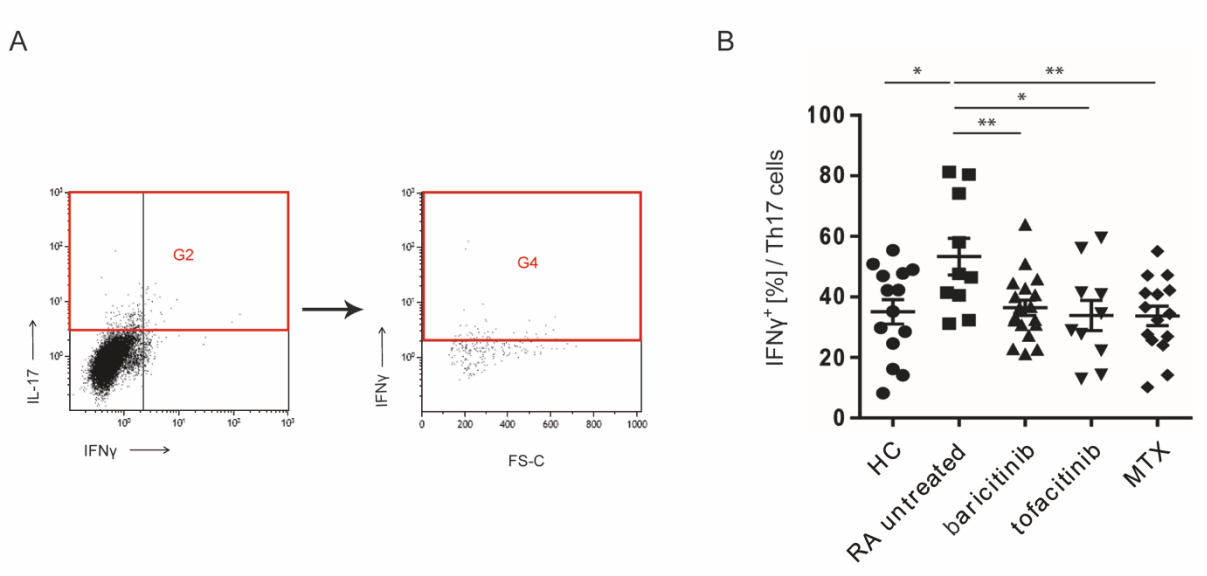


**Figure 10: The percentage of IL-17$^+$IFNγ$^+$ cells within the Th17 cell population is significantly increased in patients with untreated RA and reduced after treatment.** Flow cytometry analysis of the double positive Th17 cells (Th17.1) within the Th17 cell population. **A)** Th17 cells were selected by gate G2 and displayed in a new graph. The percentage of IFNγ$^+$ cells within these selected Th17 cells was analyzed by gate G4. **B)** The percentage of Th17.1 cells from Th17 cells is significantly increased in untreated RA patients. Treatment with baricitinib, tofacitinib and MTX significantly decreases the proportion of IFNγ expressing Th17 cells. HC n= 12, RA untreated n=9, baricitinib n=18, tofacitinib n=10, MTX n=15. *$p$ < 0.05, **$p$ < 0.01. P-values were calculated using one-way ANOVA. Data are shown as mean ± SEM.

## 5.3 Treatment with baricitinib and tofacitinib does not restore $T_{reg}$ cell frequencies

Regulatory T cell frequency in peripheral blood was analyzed by flow cytometry. Due to the definition of $T_{reg}$ cells as CD4$^+$CD25$^{hi}$CD127$^{low}$ cells, CD4$^+$ T cells were stained with extracellular antibodies anti-CD25 PE and anti-CD127 FITC (figure 11A).

The highest expression of $T_{reg}$ cells was shown in healthy controls with a mean of 8.93% ± 0.67. Compared to healthy controls, patients with untreated rheumatoid arthritis showed a significantly reduced amount of $T_{reg}$ cells (5.33% ± 0.14, $p$= 0.0005). Further, the percentage of $T_{reg}$ cells after treatment with baricitinib and tofacitinib was at the same reduced level that was observed in untreated RA, showing a quantity of approximately 5%. Thus, not only untreated RA patients, but also baricitinib ($p$=0.0009) and tofacitinib ($p$=0.0003) treated patients exhibit significantly decreased $T_{reg}$ cell frequencies compared to healthy controls. In contrast to JAK inhibitor treatment, treatment with MTX was able to increase the number of $T_{reg}$ cells. With a percentage of 6.98% ± 0.61, $T_{reg}$ cells were significantly ($p$=0.0378) elevated by MTX treatment compared to tofacitinib treated patients, but still do not reach the amount of $T_{reg}$ cells in healthy controls. Thus, the data reveal that MTX treatment enhances the numbers of regulatory T cells, while treatment with baricitinib and tofacitinib does not (figure 11B). These data on the frequencies of $T_{reg}$ cells after treatment with JAK inhibitors compared to MTX and other DMARDs were published in Annals of the Rheumatic diseases Nov 2019[133].

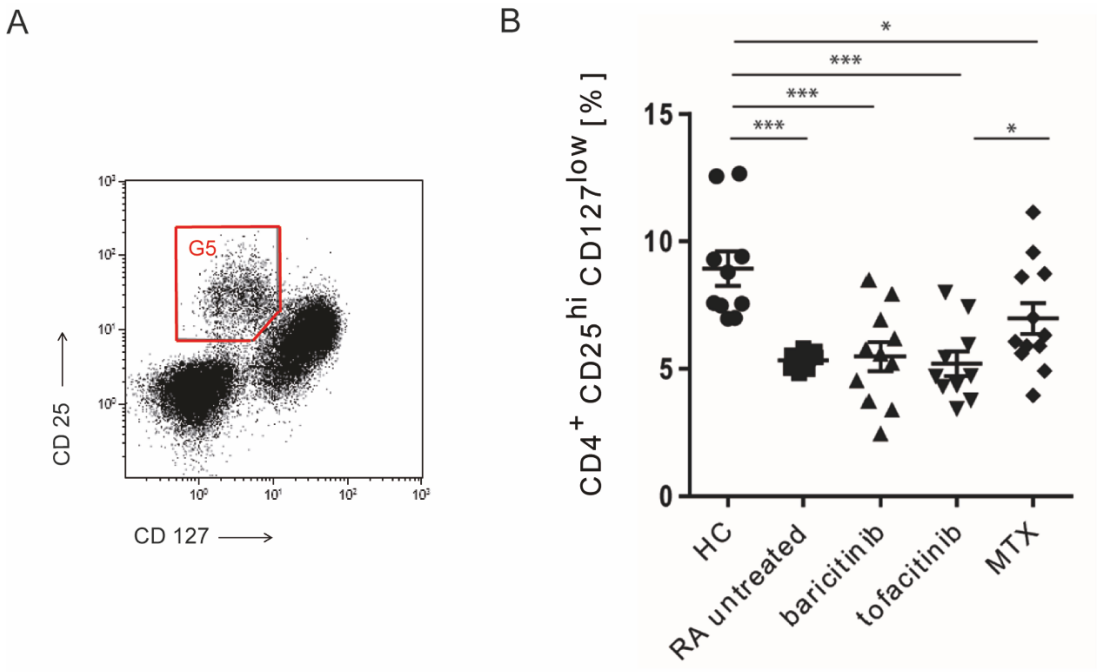


**Figure 11: Treatment with baricitinib and tofacitinib does not restore $T_{reg}$ cell frequency.** Flow cytometry analysis of $T_{reg}$ cells in $CD4^+$ T cells of peripheral blood. **A)** Gating strategy for regulatory T cells defined as $CD4^+$ $CD25^{hi}$ $CD127^{low}$. Regulatory T cells are shown in gate G5. **B**) Percentage of regulatory T cells in peripheral blood $CD4^+$ T cells. $T_{reg}$ cell frequency is strongly decreased in patients with untreated RA, as well as in patients treated with baricitinib or tofacitinib. MTX treatment does partially restore the $T_{reg}$ cell number. HC n= 10, RA untreated n=7, baricitinib n=11, tofacitinib n=10, MTX n=12. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. P-values were calculated using one-way ANOVA. Data are shown as mean ± SEM.

## 5.4 Treatment with baricitinib and tofacitinib only partially restores the Th17/$T_{reg}$ ratio

Based on the flow cytometry data of frequencies of Th17 cells and $T_{reg}$ cells the Th17/$T_{reg}$ ratio was calculated as paired sample of each patient and shown in figure 12A. While a higher ratio implies imbalance of Th17 cells and $T_{reg}$ cells in favor of pro-inflammatory Th17 cells, a lower ratio implies restored $T_{reg}$ cell frequency. The patient numbers differ from previous graphs due to missing data on $T_{reg}$ cell frequencies from samples of the early phase of the study, when focus was set on Th17 cells. Further, some data on Th17 cells needed to be excluded due to technical difficulties of flow cytometry regarding compensation and management of autofluorescence. According to figure 12A, there was an increased Th17/$T_{reg}$ ratio in patients with untreated RA compared to healthy controls ($p$=0.0025). Whereas MTX was able to significantly reduce the Th17/$T_{reg}$ ratio ($p$=0.0024), treatment with the JAK inhibitors baricitinib and tofacitinib did only partially restore the Th17/$T_{reg}$ ratio.

Figure 12B includes data of gene expression levels of Th17- and $T_{reg}$- cell associated transcriptions factors RORC and FoxP3. The relative expression of RORC and FoxP3 was analyzed by qPCR (see following figure 13) and their ratio was calculated in paired samples similar as it was performed for flow cytometry data. The ratio of RORC to FoxP3 did not show any significances and had little informative value due to very small sample numbers.

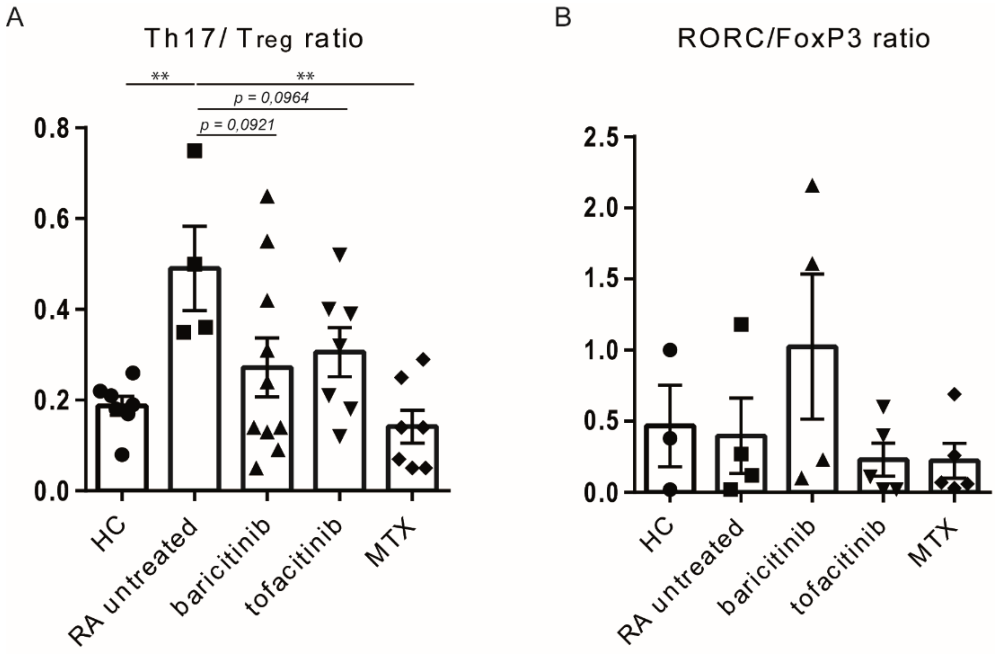


**Figure 12: The effect of baricitinib, tofacitinib and MTX on the Th17/$T_{reg}$ ratio in patients with RA.** Analysis of the Th17/$T_{reg}$ ratio based on flow cytometry (A) and RT-qPCR (B). **A)** Treatment with MTX significantly reduces the increased Th17/$T_{reg}$ ratio, while baricitinib and tofacitinib only partially restore the balance. HC n=7, RA untreated n=4, baricitinib n=10,

tofacitinib n=7, MTX n=7. **B)** The RORC/FoxP3 ratio does not show any significances. HC n=3, RA untreated n=4, baricitinib n=4, tofacitinib n=5, MTX n=5. $^{**}p < 0.01$. P-values were calculated using one-way ANOVA. Data are shown as mean ± SEM.

## 5.5 Th17 cells and $T_{reg}$ cells in gene expression level

$CD4^+$ T cells were analyzed by quantitative polymerase chain reaction regarding the expression of Th17- and $T_{reg}$- cell associated transcription factors RORC and FoxP3. Relative RORC expression was assessed in 9 patients with untreated RA, 13 patients with baricitinib treatment, 9 tofacitinib treated patients and 8 MTX treated patients as well as 8 healthy controls. They all showed low relative expression levels of RORC with mean expression between 0.53 ± 0.15 (MTX) and 1.73 ± 0.44 (baricitinib). There was no difference among the groups (figure 13A), accordingly, no indication for higher RORC expression in untreated RA. The $T_{reg}$ associated transcription factor FoxP3 was assessed in 4 untreated RA patients, 4 baricitinib treated patients and 5 patients in each other group. Highest FoxP3 expression was measured in healthy controls (5.08 ± 1.38) with significantly decreased expression levels in tofacitinib treated patients (1.06 ± 0.75; p=0.0230). Similar to the analysis in flow cytometry, the measurement of FoxP3 expression indicates a tendency of higher expression in MTX treated patients and healthy controls than in JAK inhibitor treated patients. But, due to small sample numbers, the data do not allow final statements on the effect of JAK inhibitor treatment on the gene expression of cell associated key transcription factors (figure 13B).

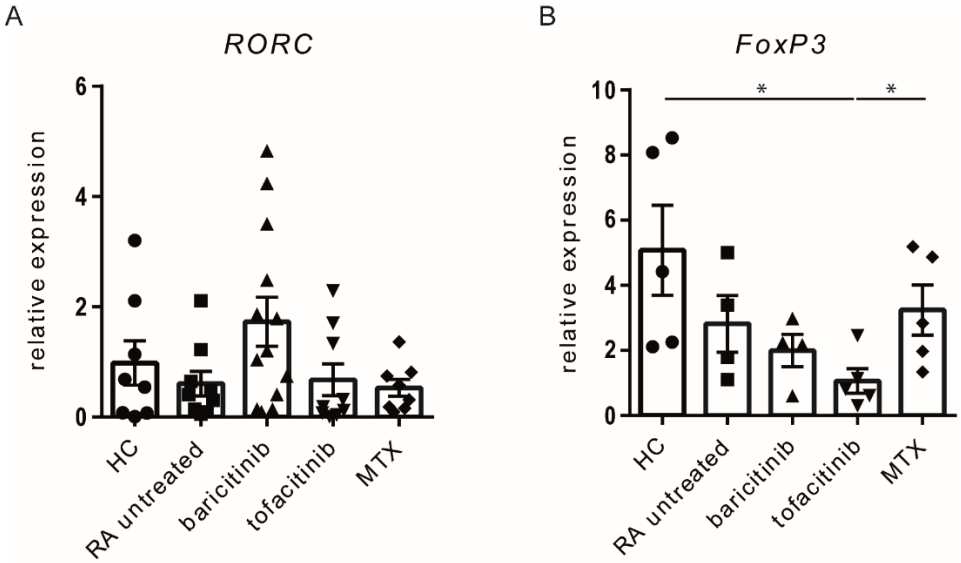


**Figure 13: Expression of Th17 and $T_{reg}$ cell associated transcription factors RORC and FoxP3.** Relative expression was assessed by qPCR from peripheral blood $CD4^+$ T cells. **A)** Highest expression of *RORC* was measured in baricitinib treated patients, lower expression in untreated RA, tofacitinib and MTX without significant differences. **B)** FoxP3 expression in tofacitinib treated patients is significantly reduced compared to MTX treated patients and HC**.** $^{*}p < 0.05$. P-values were calculated using one-way ANOVA. Data are shown as mean ± SEM.

## 5.6 Increased Th1 cells in untreated RA

Th1 cells defined as IFNγ producing $CD4^+$ T cells express the cell specific transcription factor T-bet which is encoded by the *TBX21* gene. Th1 cells were analyzed by flow cytometry and qPCR in order to evaluate treatment dependent changes in molecular levels of cell activity.
Flow cytometry analyses on Th1 cell frequencies were performed via detecting $CD4^+IFNγ^+$ T cells according to the gating scheme figure 14A. The results show similar expression levels in untreated RA (n=9), healthy controls (n=12), baricitinib (n=19) and tofacitinib treated patients (n=10). With a mean expression of 2.25 ± 0.55, IFNγ is significantly reduced in MTX treated patients compared to untreated RA patients ($p$=0.0162). Thus, MTX might have a stronger suppressing effect on the IFNγ production than JAK inhibitors have (figure 14B).
Analyzing the Th1 associated transcription factor TBX21 in gene expression level, highest expression was detected in patients with untreated RA (2.40 ± 1.19), while lowest expression was measured in baricitinib treated patients (1.14 ± 0.22). Although the relative expression of *TBX21* showed similar trends compared to the flow cytometry data of Th1 cells, a certain statement cannot be made, because of missing significances and high error bars (figure 14C).

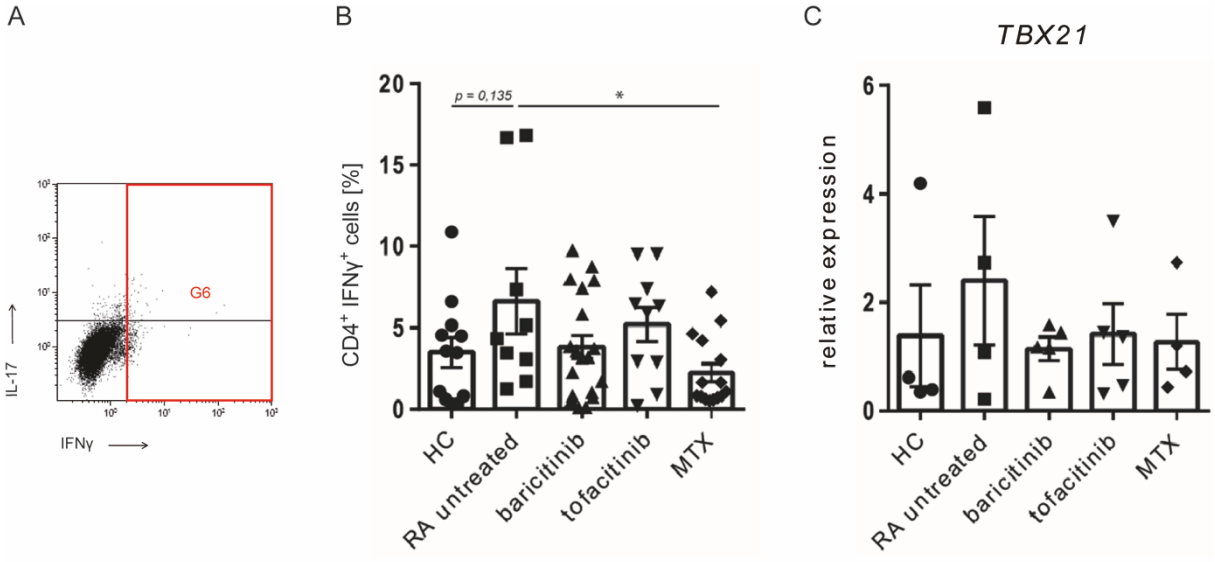


**Figure 14: Th1 cells in RA patients. A)** Flow cytometry. Gating scheme for Th1 cells defined as $CD4^+IFNγ^+$, shown in gate G6. **B)** Percentage of $CD4^+IFNγ^+$ T cells (Th1 cells) measured by flow cytometry. Compared to untreated RA, Th1 cells were significantly reduced by treatment with MTX. No significant effect after treatment with baricitinib and tofacitinib. **C)** Th1 cell transcription factor encoding gene *TBX21* was analyzed by qPCR. Highest relative expression was shown in untreated RA patients without significant differences among the different groups. *$p$ < 0.05. P-values were calculated using one-way ANOVA. Data are shown as mean ± SEM.

## 5.7 Janus kinase expression in baricitinib and tofacitinib treated patients

In order to evaluate, if drug induced inhibition of janus kinase activity also reduces JAK expression in gene transcription level, the relative expression of kinase encoding genes *Jak1, Jak2, Jak3* and *Tyk2* in $CD4^+$ T cells was analyzed by quantitative PCR (figure 15). While baricitinib was described to inhibit mainly JAK1 and JAK2, tofacitinib was described as a pan-JAK inhibitor with highest affinity to JAK3 and JAK1 and less affinity to JAK2 and Tyk2.

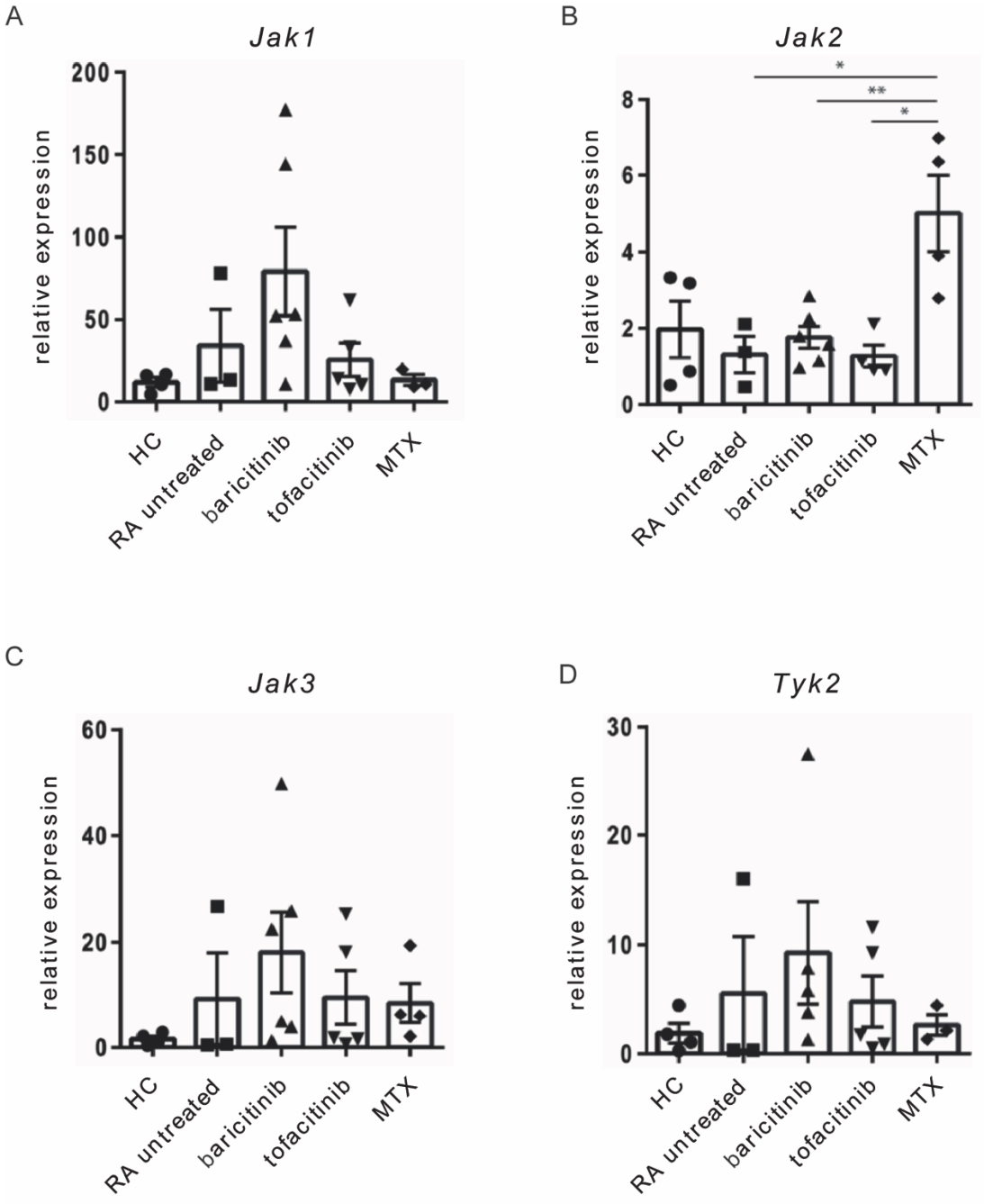


**Figure 15: Gene expression levels of janus kinases *Jak1, Jak2, Jak3* and *Tyk2* in peripheral blood $CD4^+$ T cells.** Janus kinase expression was analyzed by qPCR. **A)** Highest *Jak1* expression was measured in baricitinib treated patients, lowest *Jak1* expression in HC.

**B)** *Jak2* expression is significantly increased in MTX treated patients compared to untreated RA patients, and tofacitinib or baricitinib treated patients**. C)** HC show general rare *Jak3* expression levels. There is no significant increase of *Jak3* expression in patients with untreated or treated RA. **D)** No significant differences of *Tyk2* expression among the groups. **p* < 0.05, ***p* < 0.01. P-values were calculated using one-way ANOVA. Outlier test was performed with Grubbs (alpha 0.05) and outliers (n=3) were removed. Data are shown as mean ± SEM.

The relative expression of *Jak1*, *Jak2, Jak3* and *Tyk2* was analyzed in a population of 22 patients (HC n=4, RA untreated n=3, baricitinib n=6, tofacitinib n=5, MTX n=4), which was a very small number of patients. First data of these patients showed high error bars and very inconsistent values within one group, so that three large outliers were statistically removed. The high error bars still presented difficult conditions for evaluating any significant differences and making any statements. Therefore, PCR analyses on janus kinase expressions were not continued after the first 22 patients.

Nevertheless, at first glance it appears remarkable that relative expression of *Jak1* is generally in a higher range (y-axis) compared to the expression range of the other janus kinases. Next, with focus on the healthy controls, this group showed rather low *Jak* expression levels in all four graphs. These low expression levels were widely consistent and without outliers, as they were seen in the other groups. Thus, compared to healthy controls, there might be a slight trend of increased *Jak* expression in patients with RA, concerning both treated and untreated RA. Taking a closer look at the expression of *Jak2* (figure 15B) the analysis showed that *Jak2* was approximately equally expressed in baricitinib, tofacitinib, untreated RA and healthy controls (ranging between 1.27 ± 0.29 in tofacitinib and 1.97 ± 0.99 in HC), whereas it was significantly increased in MTX treated patients. Due to the clear tendency, this seems to be an issue, although the patient number is still small.

Regarding the certain effect of janus kinase inhibitors on janus kinase expression, more data will be needed to address this question.

## 5.8 Correlation between T cell frequency and clinical parameters

Since evidence for the implication of elevated IL-17 production in the development and progression of rheumatoid arthritis was provided, correlations between IL-17 serum levels and disease activity markers were addressed. In order to analyze the relation between Th17 cells and disease activity, the two inflammation parameters CRP and ESR were assessed. In a few patients additionally the DAS28 was measured including both, the number of affected joints and laboratory parameters. The assessed clinical and laboratory values were correlated with the percentage of Th17 cells by linear regression and r square (figure 16).

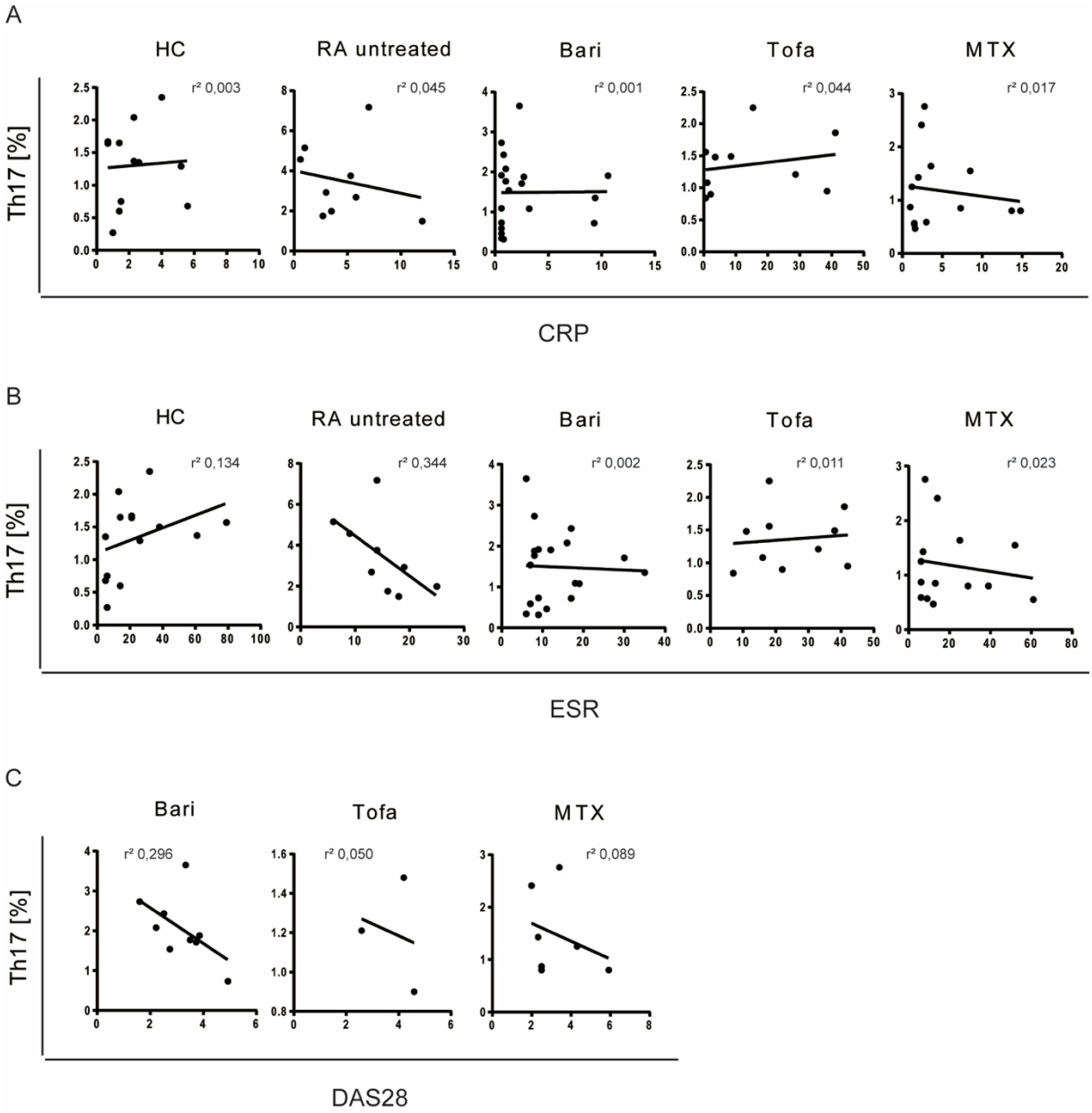


**Figure 16: Correlations between the frequency of Th17 cells in peripheral blood and the clinical inflammation parameters CRP, ESR and disease activity score (DAS28). A)** The percentage of Th17 cells is not associated with increased or decreased CRP levels. **B**) There is no significant correlation between Th17 cell numbers and the erythrocyte sedimentation rate. **C)** The disease activity score 28 assessed in baricitinib, tofacitinib and MTX treated patients is not significantly correlated with the amount of Th17 cells but indicates slightly negative correlation. HC n=12, RA untreated n=9, baricitinib n=19 (DAS28 n=9), tofacitinib n=10 (DAS28 n=3), MTX n=14 (DAS28 n=7). Correlation was analyzed by linear regression and r square.

The data show that there is no significant correlation between the amount of Th17 cells and laboratory CRP values. In all five patient groups, high and low CRP levels were associated with both, high and low Th17 cell number, causing weak r square values. While most baricitinib

treated patients show physiological CRP levels, in three patients, who could be proposed as non-responder, considerably increased CRP levels were assessed, but still do not correlate with higher percentage of Th17 cells. Tofacitinib treated patients highly range in CRP values, while the frequency of Th17 cells is relatively stable between 0.84% and 2.25% (figure 16A). Inflammatory signs were not exclusively shown in RA patients, but also in healthy controls. This is caused by the defined characteristics of 'healthy' controls as patients from the outpatient clinic without any confirmed autoimmune disease, but with other forms of arthropathy that can also be related with inflammatory signs.

Analyzing the correlation between Th17 cell numbers and ESR, no correlation could be observed in baricitinib, tofacitinib and MTX treated patients, and the linear regression displays similar orientation to CPR graphs with weak r square values. Interestingly, in patients with untreated RA, ESR seems to be slightly negative correlated with the percentage of Th17 cells ($r^2$ = 0.344), whereas healthy controls rather indicate a slight positive correlation ($r^2$ = 0.134) (figure 16B).

There was no significant correlation shown between disease activity score (DAS28) and the percentage of Th17 cells. Surprisingly, higher DAS28 seem to imply even lower Th17 cell numbers, indicating a slightly negative correlation (figure 16C). However, due to the few numbers of assessed disease activity scores, these data need to be carefully rated.

Apart from elevated Th17 cells, decreased frequency of regulatory T cells was implicated in the pathogenesis of RA. Therefore, the following figure additionally analyzed the correlation between the percentage of $T_{reg}$ cells and clinical markers for disease activity including CRP, ESR and DAS28 (figure 17). Whereas our data slightly indicate an inverse correlation between $T_{reg}$ cell frequency and CRP values ($r^2$=0.355) as well as ESR values ($r^2$=0.305) in patients with untreated RA, there was no correlation observed in HC, baricitinib, tofacitinib and MTX treated patients, neither regarding $T_{reg}$ cells and CRP (figure 17A), nor regarding $T_{reg}$ cells and ESR (figure 17B). Similar to the analyses of Th17 cell correlations, high as well as low percentages of regulatory T cells are associated with high and low inflammation parameters, displaying overall weak r square values.

The DAS28 was assessed in 2 baricitinib treated patients, 2 tofacitinib treated patients and 1 MTX treated patient, which are all shown in the same figure (figure 17C). With an $r^2$ value of 0.080 the correlation is marginal but indicates a slight inverse linear regression, as far as assessable by this restricted quantity of data.

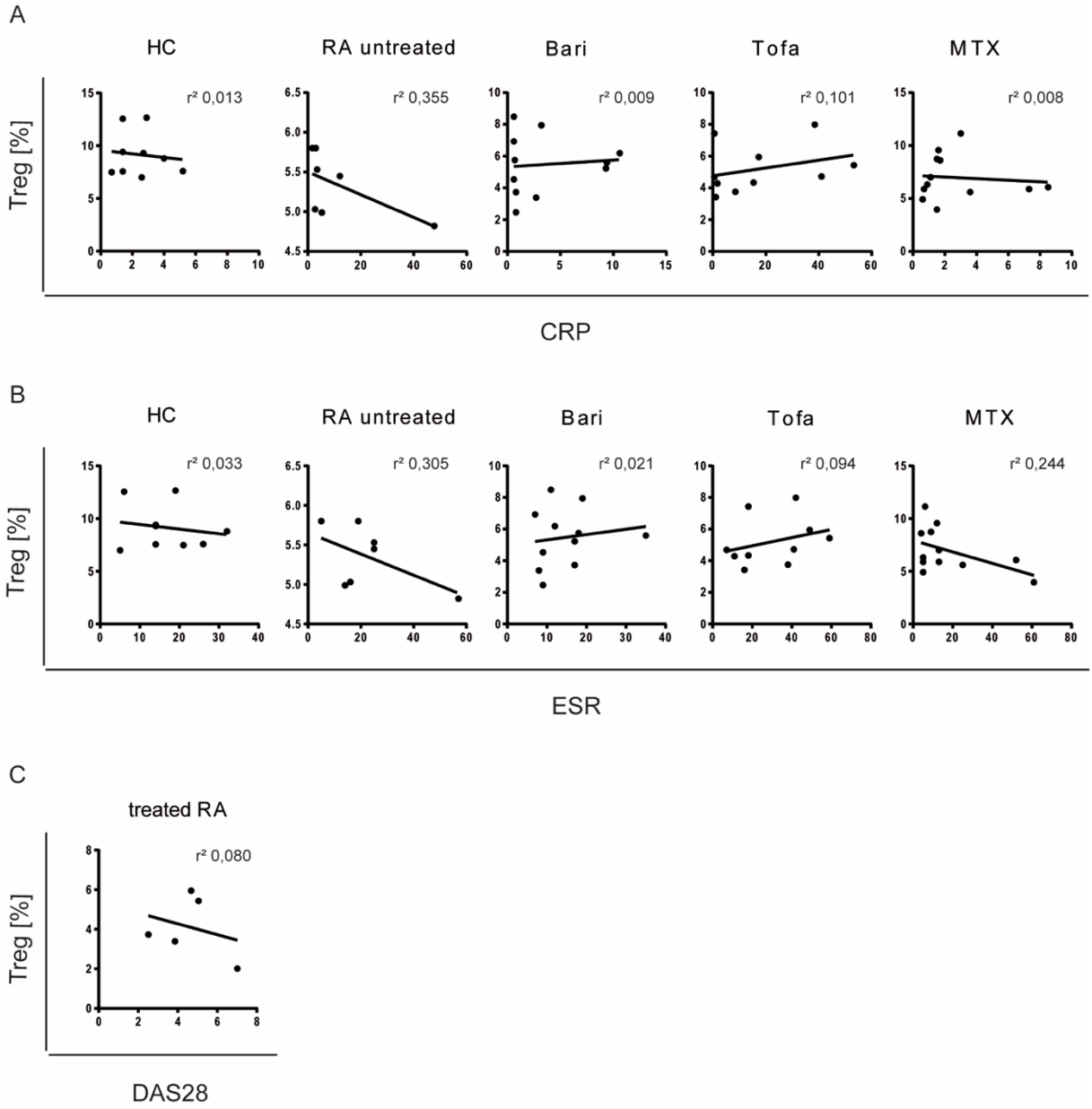


**Figure 17: Correlations between the $T_{reg}$ cell frequency in peripheral blood and the clinical inflammation parameters CRP, ESR and disease activity score (DAS28).** Inverse correlation between $T_{reg}$ cell frequency and CRP values (**A**) and ESR values (**B**) is shown in patients with untreated RA, whereas no significant correlation could be observed in the other patient groups. **C**) The DAS28 was assessed in 5 patients including 1 MTX treated patient, 2 baricitinib and 2 tofacitinib treated patients. There is no significant correlation between DAS28 and the amount of $T_{reg}$ cells. Correlation was analyzed by linear regression and r square.

# 6. Discussion

It is known that the Th17/$T_{reg}$ imbalance is strongly implicated in the pathogenesis of RA and based on this knowledge, Th17 cells and $T_{reg}$ cells gained interest as potential treatment targets. Focusing on the crucial role of the JAK/STAT pathway in the differentiation of Th17 cell and $T_{reg}$ cells, our data showed the therapeutic effect of JAK inhibitor treatment on Th17 cells and $T_{reg}$ cells with regard to the immunologic balance. We underlined the strong inhibitory effect of baricitinib and tofacitinib on pathogenic Th17 cells while pointing out a missing effect on $T_{reg}$ cells. In the following, these results and also non-significant data of our study are discussed with respect to other studies, to limitations and to potential implications in future research and improvement of RA treatment strategies.

## 6.1 Treatment effects of JAK inhibitors on Th 17 cells

As expected, our results show highly increased IL-17 secretion in patients with untreated RA, contributing to previous findings which underline the impact of Th17 cells and their pro-inflammatory capacity in the development of autoimmune diseases[49-51,67]. Further, treatment with both JAK inhibitors baricitinib and tofacitinib effectively reduces the IL-17 expression. This might be explained by the inhibition of JAK-mediated downstream phosphorylation of STAT3, which plays a crucial role in Th17 cell differentiation[134]. Concordant with our results, Maeshima et al.[135] reported decreased production of IL-17 and IFN-γ after in vitro administration of tofacitinib. The suppressive function of tofacitinib was also described by Migita et al.[136], showing inhibitory effects on a range of cytokines including IL-4, IL-17, IL-22 and IFN-γ, but not on IL-2. Thus, it can be supposed that tofacitinib not only interferes with the STAT-mediated differentiation of Th17 cells, but also with the production of proinflammatory and self-reinforcing cytokines. According to Kubo et al.[137], the mode of action of both, baricitinib and tofacitinib can be attributed to the inhibitory effect on the autocrine feedback of type-I IFN and its receptor associated kinases JAK1 and TYK2. Via inhibition of janus kinase-mediated type-I IFN signaling, JAK inhibitors seem to decrease the expression of CD80/86 on dendritic cells and thereby reduce dendritic-cell associated stimulatory capacities and proinflammatory effects. Convenient with our data, it was described that both JAK inhibitors inhibit IL-17 expression in a similar extend[137]. Therefore, we assume that the ascribed kinase affinities of baricitinib and tofacitinib are too unspecific to draw conclusions on different janus kinase downstream signaling.

A potential bias in IL-17 expression might occur by co-medication. Thus, other daily used drugs, for example simvastatin, were also reported to show effects on IL-17 expression[138]. The co-medication was not valued in this study but brings an interesting aspect. Regarding the inhibitory effect of simvastatin on Th17 cells, co-medication might possibly bring additional benefits in reducing disease activity. Apart from Simvastatin there might also be other daily used drugs with favorable effects on autoimmune disorders by enhancing the anti-inflammatory mechanisms.

Regarding that pathogenic properties of Th17 cells were mainly ascribed to their key cytokine IL-17, the development of IL-17 antibodies for RA treatment seemed likely. But interestingly, the pure blockade of IL-17 by specific IL-17 antibodies like secukinumab did not bring the expected benefit. Secukinumab failed to restrain disease activity in RA and did not show differences compared to placebo within clinical trials[139,140]. These findings question the predominant meaning of IL-17 and underline the complexity of the cellular immunologic disbalance in chronic inflammation. Interestingly, secukinumab was approved for the treatment of other systemic rheumatic diseases including psoriatic arthritis and ankylosing spondylitis, which were also associated with disruption of the IL-17 homeostasis[141]. Causes for the inefficacy of IL-17 blockade in RA remain unknown.

In respect to the plasticity of Th17 cells, the high pathogenic properties of the IL-17$^+$/IFN-γ$^+$ double positive Th17.1 cells were described above. Consistent with previous studies from Bazzazi et al.[73], we observed an overexpression of this population in patients with untreated RA, whereas both JAK inhibitors were able to significantly reduce Th17.1 cells. Therefore, it can be presumed that JAK inhibitors either might be able to break the resistance to $T_{reg}$ cell mediated suppression or might directly suppress the plasticity towards Th17.1 cells. Regarding the second point, JAK inhibitors need to be able to either reduce the expression of IL-12 and IL-23, because these cytokines mainly drive Th17 cell plasticity towards Th17.1 cells[142] or must be able to inhibit the interaction between these cytokines and Th17 cells. The certain mechanisms behind the effect of JAK inhibitors on Th17.1 cells have not been studied yet, but at this point, it would be interesting to analyze the expression of IL-12 and IL-23 in peripheral blood after treatment with JAK inhibitors.

## 6.2 $T_{reg}$ cells and their possible impact on new treatment strategies of RA

Our data on $T_{reg}$ cells support the findings of several other studies [92,98,101,111,143,144], showing decreased $T_{reg}$ cell frequencies in patients with untreated RA. Nevertheless, increased or equal numbers of $T_{reg}$ cells in RA have also been observed[94-97]. Having a closer look on the methods, all of these studies were performed by flow cytometry analyses, but distinct definitions of $T_{reg}$ cells were used. In the early studies showing rather increased $T_{reg}$ cell numbers, regulatory T cells were described as $CD4^+$ $CD25^{+/high}$ cells, whereas today we know that CD25 can be expressed on any activated T cell. Thus, to classify $T_{reg}$ cells, nowadays low CD27 expression and/or Foxp3 expression is added to the basic surface characteristics of $CD4^+$ and $CD25^{high}$.

Analyzing the effect of baricitinib and tofacitinib on $T_{reg}$ cell frequency, we showed that JAK inhibitors are not able to restore decreased $T_{reg}$ cells in RA. This essential finding was published 2019 in Meyer/Wittekind et al.[133], where we additionally compared the effect of JAK inhibitor treatment to conventional and biological DMARD treatment. We showed that MTX, as well as IL-6R blocker and TNFα inhibitors significantly increase the frequency of $T_{reg}$ cells, while baricitinib and tofacitinib do not. Having a closer look into the JAK/STAT signaling one possible hypothesis to explain this finding might be the fact that not only differentiation of pro-inflammatory Th17 cells, but also the differentiation of immunosuppressive $T_{reg}$ cells is dependent on JAK mediated STAT activation. Thus, with the inhibition of janus kinases, $T_{reg}$ cell differentiation and function appears to be also inhibited. Supporting this theory, Goldstein et al. revealed a rapid downregulation of $T_{reg}$ associated FoxP3 after *in vitro* administration of two different JAK inhibitors[145]. In contrast to baricitinib and tofacitinib, TNFα and MTX do not target the JAK/STAT signaling pathways and IL-6R blocking only affects STAT3 and STAT1 mediated differentiation of Th17 cells and Th1 cells, without interfering $T_{reg}$ cell activation. This might explain the treatment dependent differences in $T_{reg}$ cell frequencies. Confirming our data, other studies have also described a capacitating effect on $T_{reg}$ cells by treatment with MTX [144,146,147], TNF-a blocker [143,148-150] and IL-6R blocker [151,152], while abatacept treatment was heterogenous reported describing both, decreased [153,154] and increased $T_{reg}$ cell populations [155,156].

Based on the impaired $T_{reg}$ cell function in RA combined with the background knowledge on the crucial role of $T_{reg}$ cells in the maintenance of immune tolerance and prevention of autoimmune diseases, $T_{reg}$ cell-based therapies seem to be promising in re-establishing the impaired T-cell homeostasis[157-159]. Taken into account that IL-2 is the key cytokine to initiate differentiation and expansion of regulatory T cells, the administration of low-dose IL-2 has been investigated for the treatment of autoimmune diseases[160]. It potently expanded and activated

the $T_{reg}$ cell population without stimulating effector T cells and was well tolerated in a clinical phase I-IIa trial [161]. Further, Yang et al. reported that IL-2 and activated STAT5 were able to inhibit IL-17 production and Th17 cell function[162], which would also contribute to the recovery of immune homeostasis. Therefore, low-dose IL-2 is a promising therapeutic approach but it is probably ineffective in combination with pan-JAK-inhibitors like baricitinib and tofacitinib, because of their target on IL-2 receptor downstream signaling. Rather the combination with selective JAK inhibitors like the TYK2 inhibitor BMS-986165 might be efficient, because TYK2 is not involved in IL-2-receptor downstream signaling and thereby would not suppress the effect of IL-2. It would be interesting to analyze Th17 and $T_{reg}$ cells after *in vitro* administration of BMS-986165 and low-dose IL-2 in order to detect, if the favorable effect of both single drugs can be combined to potent amelioration of the Th17/$T_{reg}$ imbalance.

## 6.3 The Th17/$T_{reg}$ balance

Derived from the relative frequencies of Th17 cells and regulatory T cells, we analyzed their relation based on the Th17/$T_{reg}$ ratio in order to characterize the immunologic imbalance. Here, a higher Th17/$T_{reg}$ ratio shows a stronger immunologic imbalance. The Th17/$T_{reg}$ ratio was also used in other studies to describe the immunologic imbalance and consistent with our data, they observed increased Th17/$T_{reg}$ ratios in RA patients [100,163,164]. With regard to established treatments, data on JAK inhibitor effects on the Th17/$T_{reg}$ ratio are still rare. For most other approved drugs including MTX[165], IL-6R-inhibitors[121,166,167] and abatacept[151] a favorable effect was already reported.

According to our data, the missing ability of baricitinib and tofacitinib to enhance $T_{reg}$ cells led to an only partially restored immunologic balance after treatment. Further, as described above, the two analyzed JAK inhibitors did not show any differences in their effect on the immunologic imbalance. Consistent with our finding, Kubo et al. [137] showed that both, baricitinib and tofacitinib inhibit Th1 and Th17 cell differentiation in a similar extend. Kubo et al. also reported that there is no difference referring the inhibitory effect on phosphorylation of STAT1 and STAT3. Given this similar effect on JAK/STAT signaling and JAK/STAT-dependent T-cell differentiation, we suppose that baricitinib and tofacitinib are pan-JAK inhibitors that reduce all JAK-mediated downstream pathways, independent on the primarily targeted kinases. A recent study from McInnes [168] confirmed this presumption showing that baricitinib and tofacitinib do not inhibit an individual cytokine signaling pathway, but all JAK-dependent cytokines. Notably, the inhibition of JAK1/JAK3-dependent cytokines was shown to be slightly stronger by tofacitinib than by baricitinib, revealing a slight difference in their inhibitory capacity[168]. However, the detailed effects of JAK inhibitors on T cell differentiation and T cell function in

RA need to be further investigated. Here, especially the use of *selective* JAK inhibitors might bring new insights into the mechanisms of specific janus kinase mediated downstream signaling. Further, the use of selective JAK inhibitors is currently investigated and recent approaches are shortly mentioned in the following excursus.

## 6.4 Second generation janus kinases as promising treatment strategies in RA

With the development of second generation JAK inhibitors, high expectations were set on additional benefit of these selective JAK inhibitors compared to pan-JAK inhibitors regarding in particular efficacy and clinical safety profile. The first approved selective JAK inhibitor was upadacitinib, a JAK 1 inhibitor, which achieved EMA approval in 2019. Upadacitinib passed phase III trials showing promising results and significant improvement of clinical signs and symptoms [169-171]. Regarding the safety profile, surprisingly, upadacitinib did not differ from pan-JAK inhibitors, although for example defects in hematopoiesis were theoretically ascribed to an inhibition of JAK 2 and therefore should theoretically not occur in selective JAK 1 inhibition [128,172]. Further, the cytokine receptor inhibition profile was also shown to be very similar among selective JAK 1 inhibitors (upadacitinib and filgotinib) and pan JAK inhibitors (baricitinib and tofacitinib) irrespective of the anticipated selectivity [168,173]. This demonstrates that our understanding of the janus kinase mediated downstream signaling and JAK selectivity is still rare and the complex JAK associated effects remain to be figured out.

Additional to JAK1 inhibitors, a selective TYK2 inhibitor named BMS-986165 (or later deucravacitinib) has been designed and is supposed to be highly selective without affecting the other janus kinases [174]. BMS-986165 has shown to potently inhibit IL-23, IL-12 and type I IFN-γ receptor downstream signaling, leading to substantial reduction of STAT1 and STAT3 phosphorylation as well as reduction of pro-inflammatory cytokines IL-17 and IFN-γ [175]. Taking together these strong anti-inflammatory effects and the hypothesis that selective TYK2 inhibitors do not inhibit JAK3/JAK1 mediated activation of STAT5 and subsequent might maintain $T_{reg}$ cell differentiation, the use of selective TYK2 inhibitors might achieve better results in restoring the Th17/$T_{reg}$ balance than pan-JAK inhibitors do. But the effect of TYK2 inhibitors on the Th17/$T_{reg}$ balance remains to be investigated, especially there are no data on TYK2 inhibitors in RA treatment. Clinical phase I and phase II trials with psoriasis arthritis patients revealed a tolerable safety profile, highlighting the absence of serious adverse events and the absence of hematologic effects or elevated liver enzymes and high serum creatinine levels, which have been observed in other JAK inhibitors [176-179]. Thus, the selective TYK2 inhibitor seems to be not only efficient in anti-inflammatory effects but also superior to pan-JAK inhibitors regarding the safety profile. Therefore, studies with TYK2 inhibitors in RA should

definitely be addressed assuming additional treatment benefits. Further, *in vitro* and *in vivo* effects on the immunologic imbalance of Th17 cells and $T_{reg}$ cells are of great interest and still remain to be investigated.

Apart from that, further approaches targeting the JAK/STAT signaling in the treatment of rheumatoid arthritis are based on the inhibition of single STATs or single cytokines in order to reduce the proinflammatory activity. Interestingly, most single cytokine inhibitors like anti-IL-12/IL-23 (ustekinumab) and anti- IL-17 (secukinumab) failed to restrain disease activity in rheumatoid arthritis, although these cytokines are strongly involved in inflammation and autoimmunity [140]. Regarding the approach of STAT inhibitors, especially STAT3 was considered as a potential treatment target in RA patients, revealing its upregulation in active disease [180]. STAT3 inhibition was reported to show strong anti-inflammatory effects and potent reduction of disease activity in CIA mice[181]. It was further shown that STAT3 inhibition decreases the expression of RORyt while inducing FoxP3 and inhibiting the production of IL-6 and TNF-a [182]. Finally, targeting the JAK/STAT signaling remains a promising treatment strategy to restore altered Th17 cell- and $T_{reg}$ cell-activity, resulting in amelioration of the physiological Th17/$T_{reg}$ balance.

## 6.5 Non - significant data

In the second part of this study, quantitative PCR analyses were performed in order to address the following questions:

a) Is increased and decreased frequency of Th17 cells and $T_{reg}$ cells based on up- or downregulation of the cell-associated transcription factors RORC and FoxP3?
b) Do JAK inhibitors 'only' inhibit the activity of janus kinases by blocking JAK mediated signaling or do they further affect the gene expression of *Jak1, Jak2, Jak3* and *Tyk2*?

### 6.5.1. A. Expression of Th17 cell and $T_{reg}$ cell associated transcription factors

With regard to the expression of key transcription factors RORC and FoxP3, it was expected to see a positive correlation between cell frequency measured by flow cytometry and the expression of key transcription factors RORC and FoxP3. This expectation was based on the knowledge of T cell development, which implies that upregulated transcription factors come

along with increased cell activity, survival and differentiation. FoxP3 for example is commonly known to play a crucial role in the immunosuppressive function of $T_{reg}$ cells by stabilization of the $T_{reg}$ cell phenotype [183,184]. Thus, increased or decreased $T_{reg}$ cell numbers were expected to be associated with higher or lower expression of FoxP3.

Surprisingly, our data did not show the expected association between expression of key-transcription factors and cell frequency. There was not even a tendency towards the expected data or any other differences, so that the analyses were discontinued after the first 22 samples. Nevertheless, other studies already showed that the upregulation of RORyt mRNA and downregulation of FoxP3 mRNA in active RA indeed reflects the immunologic imbalance and can be proven with flow cytometry, qPCR analyses and protein expression via western blotting[163]. Probably, the small sample number of this study refuses evident data. But, although we were not able to reproduce our findings of flow cytometry analyses in gene expression measurements or to work out the definite effect of JAK inhibitors on RORC and FoxP3 expression, there is one interesting aspect to highlight: Treatment with JAK inhibitor tofacitinib showed significantly reduced expression of FoxP3 compared to MTX treated patients and healthy controls. This is consistent with the previously mentioned finding that JAK inhibitors do not restore the impaired $T_{reg}$ cell number, while MTX does. It indicates that downregulation of the transcription factor FoxP3 might be one reason for impaired $T_{reg}$ cell function in RA and in JAK inhibitor treatment. But still, the mode of action how JAK inhibitors interfere with the expression of transcription factors, so far, remains unknown. Further, it remains unknow, why $T_{reg}$ cell function is impaired in RA. Recent studies assume that rather post-translational modifications of the FoxP3 gene [185-188] and facilitated cell plasticity[107] are accountable than altered cytokine pattern or the inflammatory environment [189], but further research needs to be addressed to improve our understanding of these complex immunologic processes.

### 6.5.2. B. JAK inhibitors probably do not affect janus kinase gene expression

Our quantitative PCR data of janus kinase expressions showed high error bars and thereby do not sufficiently predicate expression levels. The reasons for this can primarily be found in the very small number of analyzed samples. But, although strong statements on the presented data cannot be made, related comments and thoughts are mentioned in the following.

It was expected to see higher expression levels of janus kinases in untreated RA patients than in healthy controls, based on the hypothesis of an upregulated JAK/STAT signaling in autoimmune disease and in inflammatory conditions. Interestingly, the expression levels of these two groups did not show significant differences. This might bring the idea, that disease associated increase of the JAK/STAT activity is rather related to the inflammatory cytokine milieu and to intense cytokine induction, than to higher JAK expression. In contrast to this

assumption, Walker *et al.* [190] reported increased levels of JAK3 expression in patients with RA, especially in seropositive RA. However, the methods of their study differ from this work. For example, they used synovial tissue instead of peripheral blood samples, focused on dendritic cells instead of T lymphocytes and analyzed JAK3 expression via cryosections and immunohistochemistry instead of RNA analyses. Thus, continuing the thought, that gene expression does not significantly differ in healthy patients or in RA patients, neither in JAK inhibitor treated patients, it might be assumed that JAK inhibitors only inhibit the kinase activity and function, but do not affect transcription levels of janus kinases. Nevertheless, the mode of action of janus kinases in patients with RA and how JAK inhibitors interfere with signaling and gene expression need to be further investigated. So far, there is little published work reporting the expression of janus kinases in autoimmune diseases and even less information on janus kinase expression in patients treated with JAK inhibitors.

However, one interesting aspect to highlight was shown in regard to the expression of *Jak2*. Here we found that *Jak2* expression is significantly increased in MTX treated RA patients compared to untreated RA and JAK inhibitor treated patients. In respect to the JAK/STAT signaling pathways, this finding might explain one potential mechanism by which MTX is able to restore regulatory T cells. We suppose that high levels of *Jak2* facilitate the dimerization of JAK2/JAK2 and thereby induce the downstream phosphorylation of STAT5 which is mainly involved in differentiation and proliferation of regulatory T cells. In order to confirm this mechanism, further studies analyzing the MTX dependent STAT5 phosphorylation in RA patients will be necessary. Apart from this, another mechanism how MTX restores defective $T_{reg}$ cell function was described by Cribbs et al., presuming that MTX associated demethylation of FoxP3 leads to increased FoxP3 and CTLA-4 expression and thereby restores the suppressive function of $T_{reg}$ cells [146]. Thus, the effect of MTX is probably based on a range of different mechanisms, which all affect immune signaling in an anti-inflammatory manner, among other things by empowering $T_{reg}$ cell function.

### 6.5.3. Molecular T cell pattern and clinical disease activity

The last question we addressed with this study was to analyze the correlation between alterations in T cell frequency and clinical parameters for disease activity, such as CRP, ESR and the Disease Activity Score 28 (DAS28). The DAS28 is a well-established assessment in clinical use, which includes the number of affected joints, laboratory inflammation parameters ESR and CRP, and the subjective valuation of disease activity. According to the general consensus that RA disease is driven by elevated pro-inflammatory Th17 cells and impaired $T_{reg}$ cells, we hypothesized a positive correlation between the immunologic imbalance and

clinical/laboratory inflammation markers. We further expected to see reduced disease activity in JAK inhibitor- and MTX- treated patients, considering that clinical trials reported JAK inhibitor-mediated amelioration of clinical disease activity[191]. But, against this hypothesis, our data did not show any significant correlation neither between increased IL-17 serum levels or decreased $T_{reg}$ cell frequencies and increased laboratory inflammation parameters, nor between treated patients and reduced inflammatory parameters. So, according to our data, either there is no correlation, or there is a correlation, but our data were not able to show it. Larger amounts of datasets, especially including more assessments of the DAS28 will be necessary in order to draw conclusion on this.

In current literature, the data on correlation between clinical features and IL-17 expression diverge. While some studies demonstrated that the percentage of IL-17 cells is not correlated with DAS28, CRP and ESR [67] or radiographic parameters [98], others indeed reported a positive correlation, proposing IL-17 as an additional marker for disease activity and inflammatory activity [69,70,192]. Thus, it remains unknown, if IL-17 might be a useful marker for the evaluation of disease severity. Further, due to the high patient's diversity, IL-17 might probably not serve as an absolute marker for disease activity, but rather as a follow-up parameter to determine drug response.
Regarding the correlation between $T_{reg}$ cells and clinical inflammation markers, an association has been reported by Kikuchi et al. showing that increased circulating $T_{reg}$ cells correlate with clinical response after treatment with the IL-6 receptor blocker tocilizumab [152]. Further, Nguyen et al. proposed that the numbers of $T_{reg}$ cells suit as a potential biomarker for treatment response in RA[149]. Although the benefit in clinical use of detecting $T_{reg}$ cell frequencies or Th17 cell frequencies as biomarkers for disease activity of RA remains questionable, the progressive understanding of the pathogenesis of RA will once bring more accurate and suitable predictive markers and biomarkers which might allow an earlier diagnosis and an earlier perception on treatment response/non-response.

Reflecting additionally the subjectively experienced pain as key symptom of reduced life quality in RA, it was reported that pain intensity does not alone correlate with clinical markers of disease activity but is also associated with non-inflammatory mechanisms[193]. This theory is based on the finding that baricitinib achieves stronger pain control in RA compared to TNF-a blocker adalimumab, although the changes in clinical markers of inflammation were similar[194]. Hence, establishing reliable molecular and clinical markers to monitor disease activity might further be improvable.

## 6.6 Limitations of the study

First of all, the number of analyzed samples – especially in mRNA analyses – was too small to reveal significant effects of JAK inhibitor treatment on gene expression levels. Thus, expanded patient analyses will be necessary to finally conclude, if JAK inhibitors do affect the expression levels of janus kinases, or if they 'only' inhibit janus kinases. Moreover, the relative expression of janus kinases and transcription factors showed high differences among the measured PCR plates, which led to high error bars. Here, more a bigger amount of data could reduce high error bars.
The inconsistent numbers of data in the analyses of Th17 cells and $T_{reg}$ cells arouse from the steadily progressing experimental setup. In the beginning, focus was set on Th17 cell frequencies and blood samples were only analyzed regarding the IL-17 expression. Later, literature on $T_{reg}$ cells and the Th17/$T_{reg}$ ratio arouse interest and we additionally started the detection of $T_{reg}$ cells with focus on the immunologic imbalance.

Due to a lack of capacities in the assessment of the disease activity score DAS28 in the immunologic outpatient clinic, the correlation analyses of immunologic response (immune cell frequencies) and clinical disease activity did not show any conclusive data. Thus, the DAS28 should have been assessed in more patients. Including the DAS28 might further help to distinguish clinical responder and non-responder and with this knowledge, it could have been analyzed if clinical treatment response is associated with the immunologic balance of Th17 cells and $T_{reg}$ cells.

Last, the pure descriptive work of this study limits conclusion about JAK inhibitor functioning and detailed JAK inhibitor associated mechanisms in cell signaling. In order to reveal these mechanisms, performance of more functional analyses would be required. For example, analyzing the phosphorylation of STAT3 and STAT5 by western blotting, which would show the janus kinase activity based on the potential to induce certain downstream STATs. STAT3 and STAT5 would be the most interesting STATs, because of their crucial role in the differentiation of Th17 cells and $T_{reg}$ cells.

## 6.7 Outlook

Complementary to the performed experimental setup, next step would be to focus on the JAK inhibitor mediated effects on STAT phosphorylation in order to gain a better understanding of the JAK/STAT signaling and its biological consequences. Thereby, following questions should be addressed: Do JAK inhibitors reduce phosphorylation and activation of STAT3 and STAT5?

Is reduced expression of pSTAT3 associated with reduced Th17 cell differentiation? Is reduced expression of STAT5 associated with the inability of JAK inhibitors to restore $T_{reg}$ cell frequencies? In which way do JAK inhibitors affect the expression of other STATs? Are differences in STAT phosphorylation associated with certain changes in the cytokine mileu? Answering these questions will be required for a better understanding of the specific mechanisms in the JAK/STAT signaling pathway. Further, extended research on the novel *selective* JAK inhibitors, including JAK1 inhibitors and TYK2 inhibitors, might improve our understanding of single kinase associated functions in immunity and immune response. Based on selective JAK inhibitor mediated molecular effects on cytokine secretion and T cell differentiation, conclusion could also be drawn on specific JAK mediated signaling. While baricitinib and tofacitinib did not show any differences within this study, we would indeed expect differences in cytokine secretion and T cell differentiation within the analyses of selective JAK1 and selective TYK2 inhibitors. Especially TYK2 inhibitors might show promising findings in RA treatment, considering that TYK2 is involved in Th17 and Th1 cell differentiation, but not in $T_{reg}$ cell differentiation. Therefore, it can be assumed that TYK2 inhibitors might be able to fully restore the immunologic imbalance due to their suppressive effect on Th17 cells, while $T_{reg}$ cell differentiation is not inhibited. Hence, research on the use of TYK2 inhibitors in RA should be addressed.

Apart from that, it would be interesting to establish follow-up analyses in order to evaluate treatment efficacy in course of disease. By follow-up analyses, it could be assessed if JAK inhibitor associated effects on the Th 17/$T_{reg}$ balance depend on treatment duration and if there are indications for a development of drug tolerance. In patients with development of drug tolerance and also in primary non-responder patients the JAK/STAT signaling should be analyzed regarding its cell associated mechanisms to evade treatment effects. The molecular analysis of responders and non-responders might further help to detect predictive marker for the individual patient benefit of JAK inhibitor treatment. In regard to the multifactorial pathogenesis of RA, it might also be interesting to take lifestyle habits, environmental factors and co-medication into account in order to reveal how they affect drug response and disease activity.

Next, as discussed above, treatment approaches with low-dose IL-2 and its effect on $T_{reg}$ cells should be included in future studies. Such studies might improve our understanding of $T_{reg}$ cell differentiation and basic IL-2 receptor/JAK1/JAK3 signaling in RA and might facilitate approaches in the implementation of $T_{reg}$ cell therapies.

## 6.8 Conclusion

Taken together, this study showed the effect of baricitinib and tofacitinib treatment on Th17 cells, $T_{reg}$ cells and the Th17/$T_{reg}$ balance. The most intriguing finding is the demonstration of low $T_{reg}$ cell numbers in peripheral blood of patients treated with baricitinib and tofacitinib. The incapacity of these two non-selective JAK inhibitors to restore regulatory T cells was also noticed in the Th17/$T_{reg}$ balance, which is only partially restored. Consistent with previous studies, we showed highly increased Th17 cells and Th17.1 cells in active disease, while both JAK inhibitors were able to reduce pathologically increased Th17 cells. Based on these results, we presume that novel therapeutic strategies with the ability to fully restore the Th17/$T_{reg}$ balance might bring additional benefit in the treatment of rheumatoid arthritis. Especially the development of selective JAK inhibitors on the one hand, and the approaches of $T_{reg}$ cell therapies on the other hand, seem to be promising and remain to be investigated regarding their effect on the Th17/$T_{reg}$ balance.

Moreover, the JAK/STAT signaling with its importance in the pathogenesis of autoimmune diseases needs to be further discovered in order to extend our understanding of immunologic processes and impaired immunologic function. Profound studies on cytokine induction, cell functionality and gene transcription will bring further insights in the function of specific JAKs and STATs and thereby can improve therapeutic approaches targeting the JAK/STAT signaling pathway.

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# 8. Supplement

## 8.1 List of figures



## 8.2 List of tables

# 9. Previously published data

Data on frequencies of regulatory T cells after treatment with JAK inhibitors compared to other DMARD treatments was already published in:

Meyer A, Wittekind PS, Kotschenreuther K, Schiller J, von Tresckow J, Haak TH, Kofler DM. Regulatory T cell frequencies in patients with rheumatoid arthritis are increased by conventional and biological DMARDs but not by JAK inhibitors. Ann Rheum Dis. 2019 Nov 19: annrheumdis-2019-216576. doi: 10.1136/annrheumdis-2019-216576. Epub ahead of print. PMID: 31744827.