Investigating the impact of JAK inhibitor tofacitinib on the CD4$^+$ T cell-dendritic cell interactions in murine models of rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune condition manifested by synovial inflammation and joint destruction and is associated with high morbidity and mortality. While the existing biologic therapies revolutionised the management of RA, considerable unmet needs in disease management require the development of new therapeutic agents. Janus kinases (JAKs) are intracellular tyrosine kinases employed by Type I and Type II cytokine receptors and transducing the signals from a range of cytokines and growth factors. They are indispensable in mediating the signaling of inflammatory cytokines implicated in the pathogenesis of autoimmune conditions, thus present attractive targets for therapeutic intervention. Tofacitinib was the first JAK inhibitor approved for the treatment of RA, which was effective in patients refractory to existing treatments. Among its immunomodulatory mechanisms, tofacitinib was reported to impair the proliferation, differentiation, and pro-inflammatory cytokine production in $CD4^+$ T cells, both *in vitro* and *in vivo*. However, the impact of tofacitinib as well as the stage of the drug interference (priming or re-activation) on cognate $CD4^+$ T cell-dendritic cell (DC) interaction, which underlies both breakdown of self-tolerance and autoimmune response propagation in RA, remains to be elucidated.

Using the antigen-specific cell system both *in vitro* and *in vivo*, I have shown that tofacitinib treatment impaired the priming of the $CD4^+$ T cells by DCs, resulting in their diminished ability to differentiate into T helper 1 (Th1) subset and exhibit associated T-bet expression and IFNy production. This effect on $CD4^+$ T cells was observed both *in vitro* and *in vivo* and persisted upon secondary antigenic challenge. On the contrary, the antigen-experienced $CD4^+$ T cells primed in the absence of tofacitinib retained their functional capacity upon re-activation in the presence of the drug. Tofacitinib efficacy assessment in the mouse model of early RA similarly revealed that the antigen-experienced $CD4^+$ T lymphocytes, from both adoptively transferred and endogenous populations, remained unaffected by tofacitinib treatment. While JAK inhibitor had no impact on paw thickness, it induced notable (although non-significant) improvement in features of joint pathology, which, together with the absence of effect on $CD4^+$

T lymphocytes, suggested tofacitinib targeting other inflammatory cells contributing to the autoimmune response. Overall, these results have shown that tofacitinib interferes with the naive $CD4^+$ T cell differentiation into the Th1 subset, thereby indicating a mechanism by which tofacitinib might in part achieve clinical efficacy in RA patients. The antigen-specific system and early RA mouse model are warranted as useful platforms for further investigation of tofacitinib immunomodulatory mechanisms with the view of the optimization of its clinical use.

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Author's Declaration

I declare that this thesis is the result of my own work. The assistance of others with conducting experiments is indicated at the beginning of the relevant chapter. No part of this thesis was submitted for any other degree at the University of Glasgow or any other institution.

Marija Bedaj

Definitions/Abbreviations

1(2)^{O}	Primary/Secondary
γc	Common gamma chain
ACPA	Anti-citrullinated protein antibody
AIA	Adjuvant-induced arthritis
AICD	Activation-induced cell death
AICDA	Activation-induced cytidine deaminase
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BMDC	Bone marrow-derived dendritic cell
CD62L	CD62 ligand
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen-induced arthritis
CII	Collagen II
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte antigen
Ctrl	Control
DC	Dendritic cell
DISC	Death-inducing signalling complex
DMARD	Disease-modifying anti-rheumatic drug
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffer saline
EAE	Experimental autoimmune encephalitis
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay

ERK	Extracellular signal-regulated protein kinase
ESR	Erythrocyte sedimentation rate
ET	Essential thrombocytopenia
FACS	Fluorescence-activated cell sorting
FasL	FAS ligand
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FLS	Fibroblast-like synoviocytes
GAS	Gamma-interferon-activated site
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvHD	Graft-versus-host disease
GWAS	Genome-wide association studies
HAO	Heat-aggregated ovalbumin
HEV	High endothelial venule
Hi	High
HLA	Human-leukocyte antigen
ICAM	Intracellular adhesion molecule
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IS	Immune synapse
ITAM	Immuno-receptor tyrosine-based activation motif
JAK	Janus kinase
LAT	Linker for the activation of T cells
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide

Lo	Low
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
moDC	Monocyte-derived dendritic cell
MRI	Magnetic resonance imaging
NFκB	Nuclear factor Kappa B
OVA(p)	Ovalbumin (peptide)
PAD	Peptidyl arginine deaminase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PD-1	Programmed death-1 protein
PD-1L	Programmed death-1 ligand
PIAS	Protein inhibitor of activated STAT
PMF	Primary myelofibrosis
pSTAT	Phosphorylated signal transducer and activator of transcription
PTP	Protein tyrosine phosphatase
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PV	Polycythemia vera
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor-κB ligand
RF	Rheumatoid factor
ROS	Reactive oxygen species
RT	Room temperature
SCID	Severe combined immunodeficiency
SD	Standard deviation

SH2	Src homology 2 (domain)
SLE	Systemic lupus erythematosus
SMAC	Supramolecular activation cluster
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCR	T cell receptor
TCZ	Tocilizumab
Tfh	T follicular helper cell
TGFß	Transforming growth factor beta
Th	T helper cell
TNF	Tumour necrosis factor
tolDC	Tolerogenic dendritic cell
Treg	Regulatory T cell
Tyk2	Tyrosine kinase 2
U-STAT	Unphosphorylated signal transducer and activator of transcription
VEGF	Vascular endothelial growth factor
X-SCID	X-linked severe combined immunodeficiency

1 Introduction

1.1 Rheumatoid arthritis

1.1.1 Definition

Rheumatoid arthritis is a chronic inflammatory autoimmune condition characterized by inflammation of the joint synovial membrane (synovitis) and progressive degradation of articular cartilage and bone erosion, manifesting in chronic joint pain and progressive deterioration of physical function. Articular symptoms are accompanied by constitutional symptoms and extra-articular (systemic) co-morbidities which contribute to diminished quality of life, increased disease severity and mortality.

1.1.2 Clinical manifestations

Rheumatoid arthritis characteristically presents as a polyarticular disease with a symmetrical pattern of joint involvement, although an asymmetric/monoarticular arthritis can also occur at early presentation. The disease has a gradual onset and patients typically present with joint pain and swelling, alongside stiffness, which occurs after prolonged periods of inactivity (e.g. in the morning) and typically lasts for an hour or longer. Some patients on onset exhibit palindromic rheumatism, manifested by recurring acute self-limiting episodes, with up to two-thirds developing a chronic disease form.[1,2]

The swelling typically affects small joints of the hands and feet (including metatarsophalangeal, metacarpophalangeal and proximal interphalangeal joints) and wrist joints. Although more rarely affected, larger joints such as elbow, shoulder, ankle, and knee may also be involved. Distal interphalangeal joints are largely spared in RA. The swelling appears tender (‘soft’) due to the inflamed synovial joint membrane (synovitis) and fluid in the joint (effusion). When digits are involved, the swelling is usually focused around the joint (fusiform) without extending to the whole digit.[3]

Constitutional symptoms can also arise, which are non-specific for RA but are carefully considered during differential diagnosis. Fatigue is present in nearly all patients, with low-grade fever, low mood/depression, loss of appetite and weight loss also predominant at the disease onset.[1] Extra-articular complications/manifestations are also evidenced to develop in approximately 40% of patients and associate with more active/severe disease, thus requiring more aggressive treatment and close monitoring.[4,5] The most common manifestation is rheumatoid nodules, found in as many as 30% of patients and associated with disease severity and worse prognosis[6,7]. The fibrinoid nodules are firm lumps typically present subcutaneously next to extensor surfaces (e.g. knuckles, elbows), but can also occur at internal sites such as lung or heart[1,8,9]. The nodules are normally painless and asymptomatic, but can also compromise function, ulcerate, and become infected. Rheumatoid vasculitis is a rare but potentially most severe extra-articular feature, manifested in patients with a long-term nodular seropositive RA.[10] Vasculitis is a necrotizing inflammation of the small and medium-sized arteries, mostly involving the skin and peripheral nerves. The majority of patients develop subcutaneous vasculitis, manifested by purpura, ulcers and digital necrosis, but the involvement of major organs systems (cardiac, renal, nervous), although less frequent, can notably contribute to disease severity and mortality[11].

Patients with established RA can also present with multiple (2-3) comorbidities simultaneously. Among those, cardiovascular disease is the most common and is associated with excess mortality (by 50%) compared to the general population[12]. The development of atherosclerosis-related cardiovascular conditions in RA is potentiated by chronic inflammation and other RA-associated factors alongside the conventional cardiovascular risk factors[13]. Other frequent comorbidities include infections (particularly opportunistic), osteoporosis, pulmonary, gastrointestinal and neurological disorders[14,15,8,16,17]. The susceptibility to these similarly results from a combined contribution of traditional and RA-linked risk factors, as well as the presence of extra-articular manifestations or specific treatment regimen[16,18]. Apart from increasing mortality, comorbidities can impact the accuracy of the diagnosis, the overall patient (functional) performance and tolerability of certain antirheumatic drugs, thus their

monitoring, management, and prevention, if possible, is important in the overall treatment of RA.

1.1.3 **Impact on patient quality of life**

Considering the burden of the above constitutional and disease-specific symptoms, the health-related quality of life (HRQoL) is evidently compromised in RA patients when compared to the general population.[19] HRQoL is a multifactorial concept encompassing/overarching physical, mental and social domains of individuals' well-being, and represents a valuable measure of disease clinical state and the treatment outcome from a patients' perspective.[20,21] The major determinants impairing the quality of life in RA sufferers appear to be diminished physical function, pain, fatigue, sleep disturbances and depression. The compromised physical function leads to reduced productivity in the workplace, and an estimated 44% of RA patients permanently lose their working performance, particularly if involving physically demanding work, within 10 years from the diagnosis.[22] However, the adjustments of working conditions may allow a substantial proportion of patients to remain employed. [23] The majority of personal and instrumental daily tasks (e.g. shopping, driving, household chores) also become progressively difficult to perform due to physical limitations, which leads to loss of independence and sense of control. The alterations in both work and daily activities, along with the limitations imposed on leisure/recreation, have a hugely detrimental impact on patient's self-identity, their role in a family and social engagement.[24]

Pain, one of the key symptoms of RA, is another major concern among patients, and despite one of the therapeutic endpoints of most antirheumatic compounds being pain alleviation, the substantial levels of residual pain are nevertheless reported. The pain was found to increase with disease duration, while its incidence positively correlates with the older age of the patients.[25] A history of depression appeared to predispose patients to experience higher levels of pain, while the pain itself likely contributed to the RA-associated sleep

disturbances.[26,27] Patients indicated that pain, similarly to aggravated physical function, imposed limitations of their everyday activities and participation in any form of exercise/sport, thereby making them feel 'frustrated', 'angry' and 'helpless'.[24,28] In line with this, in a cohort of RA patients, the pain was rated first among the factors affecting the quality of life. Fatigue is another common and debilitating symptom estimated to affect >80% of RA patients on a daily basis, with 40% of cases accounting for clinically relevant fatigue.[29] RA-associated fatigue was found to most strongly correlate with altered physical functioning, pain, and depression, and appeared to increase with symptom duration and lack of social support, suggesting its strong association with psychosocial variables and not simply a clinical disease status.[30,31] The impact of targeted treatment on the fatigue is contradictory, and while some studies demonstrate the persistence of (baseline) fatigue levels despite clinical disease improvement, other research indicates a small to moderate significant alleviation of fatigue following biologic therapy.[32,33] In a qualitative study evaluating patients' perception of RA-associated fatigue, participants described the fatigue as 'overwhelming', 'unresolving' and unpredictable. The consequences of fatigue were reported to put restrictions on all spheres of individuals' life (activities, relationships, emotions), and the patients were attempting to cope using various self-management strategies (pacing, rest, persistence) with varying success.[34] The importance of fatigue was further reflected by it being identified among the key domains requiring improvement in order to achieve patient-perceived remission.[35]

The experience of physical disability/limitations, pain and fatigue inevitably impacts the mental state of the RA patients, with the prevalence of depressive symptoms ranging from 6.4% to 41.5% in affected individuals.[35] Depression is associated with increased health care utilization, reduced adherence to treatment regimens and poorer clinical responses to treatment.[36,37] Remarkably, comorbid depression appeared to independently increase the risk of mortality in RA patients.[38] A causal relationship was suggested between pain and depression, with severe pain leading to worsened mental state, and depression reciprocally worsening the pain experience.[39] Patients reported depressive episodes to be more common during the early course of the disease and related primarily to decline in physical function and the associated loss of valued activities, as well

as uncertainty about the future.[24,40] Expectedly, depression results in a more negative perception of illness and impaired coping strategies, together with a hopeless outlook on the possibility of cure.[41] Thus, depression appears to be interlinked with all other major factors influencing RA-associated quality of life and represents a significant burden for the patient's overall well-being. Hence, a number of factors aside from physical function appear to significantly influence the quality of life of RA patients and should be carefully considered during both diagnosis and assessment of the patient-centred treatment outcome. The management of these symptoms, either therapeutic or palliative, should also be considered where possible.

1.1.4 **Diagnosis**

The identification of RA at the initial presentation and a rapid aggressive therapeutic intervention at the early disease stage is extremely important since it can determine the subsequent course of disease progression, prevent joint damage, and potentially allow to achieve remission. However, this approach presents with a number of challenges, including a considerable heterogeneity of the presentations of early synovitis and the subsequent disease course, as well as the administration of unsuitable treatment to the large proportion of patients lacking definitive RA diagnosis, which is both costly and unsafe. Despite current advances and arising challenges in the disease understanding, the staple diagnostic tools remain limited and are briefly discussed below.

1.1.4.1 ACR/EULAR Classification criteria 2010

No specific diagnostic criteria have been established for early (undifferentiated) arthritis, and the joint symptoms at the onset are also often difficult to distinguish from the other types of inflammatory polyarthritis. Until recently,

the classification criteria from 1987, developed by the American College of Rheumatology (ACR) for stratification and standardizing of the RA patient groups recruited for clinical trials and based predominantly on the symptoms from long-term patients, was also widely employed for diagnostic purposes[42]. However, this approach lacked the power to provide differential RA diagnosis and discriminate patients at the disease onset that would benefit from early intervention/treatment. Thus, the criteria were revised by ACR in collaboration with European League Against Rheumatism (EULAR) in 2010, with the intention of improving the identification of patients with early undifferentiated RA that are at risk of developing the persistent/erosive disease[43].

According to the 2010 ACR/EULAR classification, a definitive RA requires the presence of at least 1 joint with clinical swelling (synovitis), the absence of an alternative condition that may better explain synovitis and a total score of ≥6 (out of maximal 10) after combining the individual scores from 4 domains (Table 1-1). The domains include: number and distribution of involved joints (0-5 points), serological abnormality (presence of RF, ACPA or both antibodies) (0-3 points); elevated levels of one of acute phase reactants (erythrocyte sedimentation rate(ESR) or C-reactive protein (CRP)) and symptom duration of ≥6 weeks each contribute a single point[43]. The updated diagnostic criteria were repeatedly verified to have a higher sensitivity for recognising patients with early RA when compared to the older classification system[44,45]. Importantly, the patients with established diseases can also benefit from the revised classification, as it has the inclusion criteria for patients with the history of erosive disease characteristic of RA, as well as patients with long-term inactive disease and a history fitting the required criteria.

Table 1-1. Classification criteria for definitive RA diagnosis*

Classification criteria **	Points
Domain A: Joint involvement (0-5 points)	
1 large joint	0
2-10 large joints	1
1-3 small joints (regardless of large joint involvement)	2
4-10 small joints (regardless of large joint involvement)	3
>10 small joints (≥1 small joint)	5
Domain B: Serology (0-3 points)	
Negative RF *AND* negative ACPA	0
Low-positive RF *OR* low-positive APCA	2
High-positive RF *OR* high-positive ACPA	3
Domain C: Acute phase reactants (0-1 points)	
Normal CPR *AND* normal ESR	0
Abnormal CRP *OR* abnormal ESR	1
Domain D: Symptom duration (0-1 points)	
< 6 weeks	0
≥ 6 weeks	1

*Adapted from the updated RA classification criteria developed by American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) in 2010[43]

**Patients eligible for testing based on above criteria must have at least 1 joint with active clinical synovitis (swelling) in the absence of an alternative condition that might better explain the synovitis

1.1.4.2 Serological markers

Serological assessment of both specific acute phase reactant and autoantibody levels is a part of the ACR/EULAR 2010 diagnostic approach. The increased levels of acute phase proteins, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), most accurately indicate the ongoing inflammatory response. Although not specific for a particular disease, the elevated CRP levels in RA closely correlate with clinical disease activity and radiographic damage progression[46,47]. Unlike ESR, changes in CRP levels indicate more recent changes in disease activity, and therefore also have a strong predictive value of the early response to treatment[47,48].

Rheumatoid factor (RF) and anti-citrullinated peptide antibody (ACPA) (more often termed anti-cyclic citrullinated peptide (anti-CCP)) antibodies are autoantibodies commonly used as diagnostic and prognostic markers in RA. RF is

an antibody recognising the crystallizable (Fc) fragment of the human IgG molecule. RF is detected in 60-90% of patients with established disease but only in half of the patients with early RA. RF can also occur in other autoimmune and infectious diseases and malignancies and occasionally detected in healthy controls, thus not being exclusively specific for RA. However, despite its moderate specificity (85%), high titres of RF are strongly predictive of the worse prognosis of erosive disease [48].

Anti-CCP antibodies are autoantibodies recognising citrulline residue on the peptides and proteins and are long thought to be implicated in the pathogenesis of RA[49]. In line with that, anti-CCP antibodies exhibit high specificity (>90%) for RA at initial presentation/early RA and can be detected, particularly in older patients, prior to the clinical disease onset.[50,51,52] Similarly, seropositivity for anti-CCP in patients with undifferentiated inflammatory arthritis can confer susceptibility for the RA development, particularly in genetically predisposed individuals.[53] Anti-CCP antibodies are highly specific as a diagnostic tool for RA and can help to differentiate RA from other inflammatory arthritic conditions. The presence/high titres of anti-CCP at diagnosis was shown to significantly correlate with higher disease activity and poor prognosis (development of erosive RA).[54] Overall, the detection of both RF and anti-CCP in the patient serum enhances the diagnostic specificity.[55]

1.1.4.3 Imaging

Together with clinical examination and laboratory assessment, imaging is the third essential part of the diagnostic process. The conventional imaging modality is radiography, performed on all patients on the first medical assessment and thus allowing the follow up on the disease progression. The benefits of this method include its universal availability, low costs, and high reproducibility. The early-stage features detectable by radiography are soft tissue swelling and juxta-articular osteoporosis, detected with most sensitivity in wrists, metacarpophalangeal and metatarsophalangeal joints, however, the technique

has limited sensitivity for identifying early inflammatory bone involvement and bone damage, important for the definitive RA diagnosis. The characteristic radiographic lesions, such as joint space narrowing, established bone erosions and joint malalignment, tend to appear later in the disease course.[56]

Upon the emergence of the potent and costly therapeutics (such as anti-tumour necrosis factor (α-TNF) agents), there was an increased demand for improving the diagnosis of patients with early aggressive RA, who would benefit from an early (therapeutic) intervention[57]. Thus, ultrasonography (ultrasound sonography) and magnetic resonance imaging (MRI) were employed as more sensitive modalities for assessing joint changes in RA. While ultrasonography allows detecting both joint inflammation (synovitis) and structural changes in the joint (including tenosynovitis, bursitis and to some extent cartilage damage and bone erosions) with precision superior to clinical assessment, it has low reproducibility and the disease activity scoring has not yet been standardized for this method.[56] MRI is the most sensitive technique to date, being superior to ultrasound (and radiography) in providing a three-dimensional view of the affected area and detecting subtle changes with the aid of contrast agents, thus allowing to predict the risk of joint damage development prior to its clinical appearance. Importantly, MRI enables to detect bone oedema, which is a strong independent predictor of subsequent bone erosion[58]. Lastly, semi-quantitative and quantitative measurements of synovitis can be performed by contrast-enhanced MRI, thus providing additional details about disease activity[59].

1.1.5 **Epidemiology**

Rheumatoid arthritis is one of the most common chronic inflammatory disorders, with an average prevalence of 0.5-1% reported among several European and North American Caucasian populations.[60,61,62,63] Studies in Southeast Asian and rural African populations have demonstrated the lowest disease occurrence (0.1-0.3%) while Native American groups (Pima and Chippewa Indians) had the highest detected incidence of RA (up to 6.8%).[64,65,66,67] Gender appears to affect

susceptibility to RA, with women two to three times more likely to develop a disease, possibly due to the effects of oestrogen on the immune and synovial cell function.[68,69] However, female reproductive hormones may also be protective, as the use of oral contraceptive pill and pregnancy) are both linked to the decreased risk of RA development.[70,71] Studies assessing familial recurrence risk in (co-)twins of an affected individual (proband) across several twin cohorts showed the increased concordance rates between monozygotic twins.[72,73] In line with this, the family history of RA is considered one of the most notable risks for disease development, which, however, is relatively modest compared to other autoimmune disorders, such as type I diabetes and multiple sclerosis. The above facts suggest the importance of genetic component in the susceptibility to RA, which will be discussed further. While age represents another risk factor, with the disease onset being common at the middle age (around 45 years of age), RA occurs at the age range spanning from 16 years old to elderly age (75 and beyond).

1.1.6 Risk factors

1.1.6.1 Genetic factors

Rheumatoid arthritis has a complex/multifactorial aetiology, with the overall genetic contribution to the disease susceptibility estimated to be 60% and the remaining input conferred by environmental factors.[72] Pioneering observations suggested a strong association between RA susceptibility and certain alleles of human leukocyte antigen (HLA) HLA-DR, over-represented in RA patients. With the advent of modern DNA sequencing techniques, Gregersen et al. in 1987 identified a 5 amino acid motif (in positions 70-74) within the third hypervariable region of the HLA-DRB1 gene, encoded by several distinct DRB1 alleles and thereby termed a 'shared epitope'.[74] This shared amino sequence was found to form part of the peptide-binding groove of HLA-DRβ1 molecule, leading to the assumption of antigen presentation playing a significant role in the disease pathogenesis. The most well-established risk-conferring alleles

encoding shared epitope motifs are members of *HLA-DRB1*04* allele group (including **0401, *0404, *0405, *0408), HLA-DRB1*0101 or *0102 and HLA-DRB1*1001 alleles, with* the amino acid sequence most common in the Caucasian population, QKRAA, encoded by *HLA-DRB1*0401* allele.[75] A unique feature of these susceptibility alleles in RA, in contrast to other HLA-associated disorders, is the allele dosing effect, whereby patients carrying 2 shared epitope-coding alleles have a higher risk of developing a disease than patients with single RA-linked allelic polymorphism. In line with that, the presence of HLA-DRB1 variants, in particular 2 copies of the risk alleles, was consistently predictive of more severe erosive disease and higher premature death rates.[76,77] Similarly, certain HLA-DRB1 alleles are strongly associated with anti-citrullinated protein antibody (ACPA)- positive disease phenotype, as shared-epitope DRB1 alleles are reported to preferentially bind citrullinated (self-) peptides with high affinity.[78] Interestingly, at least one DRB1-encoded epitope was found to be protective against severe disease independently of patient antibody status, but only in the absence of joint erosion.[79]

Given the role of HLA (also known as MHC II) molecules in antigen presentation, the predominant hypothesis attempting to explain the mechanistic input of shared epitope in RA development suggested its role in pathology-associated self-peptide presentation[80] and in the shaping of T-cell repertoire[81]. However, several other autoimmune disorders with unrelated causative epitopes and distinct pathogenic profiles, including autoimmune hepatitis[81] and Type I diabetes[82], also have their predisposition associated with DRB1 allelic variants, thereby contradicting the paradigm of antigen specificity attributed to the shared epitope. Recently, du Montcel and colleagues have re-defined a 'shared epitope' concept and proposed a new classification of susceptibility alleles based on the amino acid residues present at specific sequence positions, which improved the hierarchical classification of HLA-DRB1 alleles and thus refined prediction of HLA-DRB1-linked disease susceptibility, severity and treatment outcome.[83] This advanced system might represent a tool for potentially unraveling the mechanisms underlying HLA-DRB1 allele contribution to RA development and possibly identifying the candidate arthritogenic epitopes.

The advent of genome-wide association studies (GWAS) and the subsequent trans-ethnic meta-analysis in both European and Asian populations lead to the identification of over 100 gene loci outside HLA/MHC gene region, the specific sequence variants (single nucleotide polymorphisms, SNPs) of which were associated with RA susceptibility. The relative contribution of each individual non-HLA SNP to phenotypic disease variance was estimated at 0.08%, while together with HLA susceptibility alleles they accounted for 19.5% of disease variance.[84] Among non-HLA genes, the missense polymorphism in a gene encoding protein tyrosine phosphatase 22 (PTPN22) had the strongest association with RA susceptibility, primarily in Caucasian populations.[84,85] The amino acid substitution (R620W) in PTPN22 gene results in the inability of its product, lymphocyte phosphatase (Lyp), to form a complex with its partner kinase (Csk), and thereby abrogates the Lyp/Csk synergistic capacity for effective negative regulation of proximal T cell receptor (TCR) signalling.[86,87] Other important, albeit weaker, associations were identified between RA risk and gene variants crucial for immune system function, including tumour necrosis factor receptor-associated factor-1/complement component 5 (TRAF1-C5), signal transducer and activator of transcription 4 (STAT4), CTLA-4, CD40, and peptidyl arginine deaminase 4 (PADI4).[88,89,90] Interestingly, the disease risk association with some of these genes was more pronounced in certain ethnic groups, with PADI4 polymorphisms having the strongest predictive value in Asian patient cohorts.[91] Moreover, certain non-HLA gene variants were found to be specifically linked with development of the anti-citrullinated peptide antibody (ACPA)-positive RA phenotype (e.g. IL-2RA) while others were predominantly reported in association with ACPA-negative disease (e.g. interferon regulatory factor 5, IRF5). In contrast, other gene variants (e.g. STAT4,PTPN22) were common in both disease serotypes.[92] A number of individual candidate gene-association studies identified the association between (non-HLA) susceptibility genes and RA severity in the context of radiographic joint damage, but so far only the TRAF1-C5 variant has been repeatedly reported to correlate with severity of joint destruction.[93,94,95,84]

One of the best estimates of the overall contribution of a genetic component to the RA susceptibility comes from the twin concordance studies. The degree of heritability reported in the two largest twin cohorts is very different. In the Danish cohort, the heritability in monozygotic twins was 12%, while shared and

non-shared environmental factors were estimated to contribute to RA development by 50% and 38%, respectively.[73] However, the data from Finish and UK twin cohorts suggested the genetic contribution to be much higher (53-65%).[72] Thus, while the contribution of genetic factors towards susceptibility to RA is substantial, although variable between twin cohorts, the environmental component is also implicated to be an important predisposing factor for disease development.

1.1.6.2 Microbiota

Among environmental factors, intestinal microbiota, which has a crucial role in the early shaping of the host immunity in homeostasis, is considered an important candidate contributing to the autoimmune response development in susceptible individuals.[96] Several studies in mouse models of both spontaneously occurring and inducible arthritis consistently demonstrated the animals kept in germ-free conditions to be protected from disease development. However, mono-colonization of these mice with certain species of intestinal bacteria appears sufficient to drive induction of inflammatory arthritis, by preferentially eliciting Th17 cell response.[97,98] Specifically, the introduction of *Prevotella copri* species into antibiotic-treated mice demonstrated its capacity to establish dominance within gut microbiome and subsequently exacerbate the severity of chemically- induced colitis.[99] Similar observations were made in the new-onset treatment-naïve patient US cohort, in which the RA manifestation correlated with overrepresentation of *Prevotella corpi* alongside with reduction in *Bacteroides* species.[99] While the study in the Japanese cohort partly replicated these findings[98], a metagenomic screening of the Chinese cohort gut microbiomes revealed a distinct pattern of dysbiosis, manifested by *Haemophilus* species depletion and *Lactobacillus salivarius* abundance[100]. Collectively, this data suggests that dysregulation of intestinal microbiota composition (dysbiosis) might represent an important factor underlying aberrant immune response in RA, but the universal inciting genera of symbiotic gut bacteria are yet to be identified.

Another possible epidemiological link was recently established between rheumatoid arthritis and periodontal disease. While this association is not universal, several studies so far showed periodontitis to be more prevalent in RA patients[101,102], and the treatment of periodontal disease appeared to concomitantly improve RA clinical status[103]. Among several oral bacteria known to cause periodontitis, the investigation predominantly focused on *Porphyromonas gingivalis*, since it (exclusively) encodes a peptide peptidyl arginine deaminase (PAD) which can convert arginine residue to citrulline[104] and thereby (potentially) promote neo-epitope formation. These mucosal neo-epitopes subsequently potentiate self-tolerance breakdown and production of anti-citrullinated peptide antibodies (ACPA), which represent a highly specific RA biomarker and associate with poorer disease prognosis and lower remission rates[105]. Indeed, *Porphyromonas gingivalis* was experimentally confirmed to promote PAD-mediated citrullination of human fibrinogen and α-enolase peptides[106]. Concurrently, antibody responses to *P.gingivalis* in patients with early RA repeatedly/consistently correlated with the presence (and higher titres) of disease-associated autoantibodies, including anti-cyclic citrullinated antibodies and ACPA[107,108]. Collectively, the current data support the potential contributing role of periodontal disease and specifically the inciting *P.gingivalis* species in the immune tolerance breakdown and subsequent development of RA.

1.1.6.3 Infectious agents

Apart from the resident host microbiome, infections with certain microorganisms, including viruses, bacteria, and mycoplasma, may also play a part in RA etiopathogenesis. Among those, one of the most well-characterized association is between the disease and the Epstein-Barr virus (EBV) infection. The active EBV infection was detected in the synovial tissue cells of arthritis patients, while in another study ~90% of patients, in contrast to only 8% control individuals, presented with antibodies against the rheumatoid nuclear antigen (RANA), harboured by EBV-infected B lymphocytes[109,110] Moreover, the diminished response of T cells specific for gp110, a major EBV replicative

antigen, might suggest a defective control of the infection in RA patients, resulting in the spread of the EBV antigens and thus promoting persistent synovial inflammation[111]. One of the proposed pathogenic mechanisms of EBV is molecular mimicry, which postulates that the microbial agent shares an epitope sequence with host self-proteins, thereby promoting epitope spreading and autoantibody generation. Thus, EBV glycoprotein gp100 harbours an amino acid motif homologous to that of the HLA-DRB04*01 shared epitope[111], while antibodies from RA patient serum binding to a sequence within EBV nuclear antigen-1 epitope appeared to be cross-reactive with epitopes on human keratin, type II collagen, and actin[112]. In addition, EBV antigens can also undergo citrullination and thereby represent additional targets for ACPA antibodies in patients with rheumatoid arthritis[113].

Urinary tract infection and its common causative bacterial agent, Proteus mirablis (*P.mirablis*), are also closely linked to aetiology of rheumatoid arthritis. Two independent studies described the presence of *P.mirablis* in the urine and the associated asymptomatic bacteriuria at much higher frequencies in RA patients compared to healthy controls or individuals with other autoimmune conditions including ankylosing spondylitis and osteoarthritis. The RA patients also presented with significantly elevated levels of antibodies against *P.mirablis* in both serum and urine samples when compared to age and gender-matched controls, while the antibody titres for other common causative agents of urinary tract infections (e.g. *Escherichia coli*) or intestinal commensal bacteria remained unaffected.[114,115,116,117] These antibodies were shown to recognise bacterial epitopes on haemolysin protein (ESRRAL) and urease (IRRET) of *P.mirablis,* which closely resemble the amino acid motifs present in HLA-DR1/4 molecule epitope (EQRRAA) and type XI collagen, respectively.[117] Thus, one of the suggested mechanisms of *P.mirablis* contribution to RA development might be the potentiation of cross-reactive antibody generation, which would target both bacterial and self-epitopes and feed into the cycle of joint tissue damage, neoepitope formation and further autoantibody generation.

Chikungunya, an emerging tropical arboviral infection, is widely reported to result in the development of post-chikungunya chronic inflammatory rheumatism, defined as musculoskeletal pain, unspecified arthralgia, or

arthritis, with the latter being an RA 'mimic'. Following the acute phase of infection, characterised by viremia, high-grade fever, skin rash, and polyarthritis/polyarthralgia, patients progress into the chronic phase with persistent joint involvement, with frequencies of chronic chikungunya arthritis varying among different populations/cohorts. Thus, in a Mauritius cohort examined 27.5 months post-infection 78.6 % of patients reported persistent musculoskeletal symptoms, with 5% of infected individuals fulfilling the updated ACR criteria for rheumatoid arthritis.[118] In another study a similar proportion of patients (75%) developed rheumatism one month post-infection, and following 10 months of illness as many as 36% of patients with joint pain were classified as having developed clinical form of RA.[119] The infected individuals commonly presented with morning stiffness, polyarthritis affecting wrists, ankles and knees and chronic (joint) pain, all being reminiscent of clinical RA.[119,120,121] Another indication of possible chronic arthritis development was the detection of joint effusion, bone erosion and synovial thickening in patients with chronic joint pain.[119] However, the mechanisms by which the infection causes persistent chronic inflammatory arthritis remain elusive. One of the suggested underlying mechanisms might be long-term viral persistence, with significant levels of chikungunya virus RNA detected in the tissues of non-human primates, including joints, as well as in a single patient with chronic arthritis-like pathology.[122,123] Similarly, high titres of chikungunya-virus specific IgM antibodies were observed for up to 180 days post-infection in patients with RA-like pathology and no previous history of musculoskeletal disorders prior to infection, suggesting the possible contribution of viral antigen persistence to pathology establishment.[124] The viral persistence itself is hypothesized to result from an inefficient immune response. For instance, the NK cells in patients with chronic chikungunya arthritis were reported to be functionally dysregulated due to reduction in perforin-positive NK cells along with the increased frequency of TNFα-positive NK-like T cells.[125] Another study in the chronically infected patient cohort reported the upregulation of inhibitory and downregulation of activating receptors on NK cells, with this receptor imbalance likely compromising cell anti-viral function and thus contributing to disease chronicity.[126]

1.1.6.4 Smoking and air pollutants

Cigarette smoking is an environmental factor most strongly associated with predisposition to rheumatoid arthritis development. The disease risk is positively correlated with both the amount of smoking and the duration of cigarette use, and the increased susceptibility persists even after habit cessation for 20 years or longer[127]. In particular, cigarette use is associated with ACPA-positive disease phenotype[128]. A gene-environment interaction appears to further potentiate the relative risk of RA, as the smokers carrying a single or double copy of the shared epitope (of the HLA-DRB1 allele) have an extremely elevated risk of developing ACPA-positive disease[129]. Since smoking was found to increase the extent of cell citrullination in the alveolar lavage, it might thereby contribute to the neo-epitope formation and promote the autoimmune responses to citrulline in the genetically predisposed individuals. The underlying mechanism might be explained by smoking-associated elevated lung expression levels of extracellular PAD2 enzyme, which catalyses post-translational citrullination of peptides[130]. Another mechanism might involve activation of aryl hydrocarbon receptor (AHR) by several of its ligands, present in cigarette smoke.[131] The resulting impact on the immune system include enhanced Th17 differentiation of T cells[132], dysregulated bone homeostasis and bone resorption[133], and pro-inflammatory cytokine upregulation by fibroblast-like synoviocytes, all of which can contribute to RA pathogenesis[134].

The positive lung involvement in the RA pathogenesis led to the investigation of other air-borne pollutants as potential factors potentiating susceptibility to disease. Occupational silica exposure for the prolonged duration was incriminated as a significant risk factor for RA. More recently, high exposure to certain types of pesticides in the agricultural setting among males was also linked to the increased incidence of the disease[135].

1.1.7 **Treatment**

1.1.7.1 Treatment goals and challenges

Currently, rheumatoid arthritis remains an incurable condition, but the available therapeutic armamentarium allows the efficient control of the disease. The short-term treatment goals are the reduction of joint pain and swelling, and functional improvement, while the long-term goals represent the achievement of remission in early RA, defined by the absence of disease activity, or low-disease activity in established RA, characterized by minimal remaining activity with low risk of damage progression. A treat-to-target approach is implemented to reach the above goals and comprises delivering the treatment to achieve the chosen target, assessing the intermediate response (at specific time point) and adjusting the therapy as required[136]. The improvement in composite measure of clinical disease activity of 50% or above at 3 months after treatment initiation is predictive of reaching the treatment target at 6 months, while the patients who demonstrate none or poor improvement require the change of therapy in order to achieve the treatment goal[137]. The treatment response on follow-up is assessed using composite measures, in which the total score combines information about several disease (activity) aspects, namely the number of tender/swollen joints, acute phase response and global health. While one of the composite measures, disease activity 28 (DAS28), was extensively validated in clinical trials, the clinical disease activity index (CDAI) and simplified disease activity index (SDAI) are equally sensitive and easier to use in daily clinical practice. Apart from the disease activity, the specific cut-off points of the above indices define the states of the disease and thus direct the appropriate treatment decisions[138]. However, despite the ongoing advances in the therapeutic options and treatment guidance in RA, approximately 40% of all patients remain refractory even after consecutive treatment with several biological disease modifying drugs (bDMARDs)(reviewed below), thus a new treatment strategy is urgently needed. The emerging aspiration, taking into account the heterogeneity of pathobiological and underlying immunological

profiles, is to provide a personalised therapy based on the individual patient disease endotype[139].

The main classes of current therapeutic agents employed for RA management are briefly described below and summarized (with exception of Janus kinase (JAK) inhibitors) in Table 1-2**Error! Reference source not found.**.

1.1.7.2 Conventional synthetic disease-modifying antirheumatic drugs (csDMARDs)

RA patients are treated with disease-modifying antirheumatic drugs (DMARDs), which are therapeutic agents designed to reduce disease symptoms, prevent, or reduce joint damage and maintain joint integrity and function. Conventional synthetic DMARDs (csDMARDs) were approved for RA treatment through empirical testing and include methotrexate, sulfasalazine, leflunomide, and hydroxychloroquine, used either as a monotherapy or in combination. Methotrexate is the first line DMARD used in the patients with active RA that demonstrates excellent efficacy as a monotherapy and allows to achieve sustained remission or low disease activity in nearly 50% of patients when combined with glucocorticoids[140]. Glucocorticoids or non-steroid anti-inflammatory drugs are implemented only as adjunctive therapy for initial control of inflammation between the time of diagnosis and initiation of treatment with DMARDs, or during the time required for DMARDs to initiate a response, and are only used short term due to adverse side effects.[141] Methotrexate monotherapy also demonstrates comparable efficacy to biologic DMARDs (e.g. anti-tumour necrosis factor (TNF) therapy) and, importantly, both biologic and synthetic targeted DMARDs show greater efficacy when combined with methotrexate than either of the drugs have as a monotherapy[140,142]. Conversely, the combination of methotrexate with other csDMARDs does not appear superior to the methotrexate alone, mainly due to the increased toxicity.[142] Methotrexate was designed as a competitive folate (folic acid) antagonist, which inhibits folate binding to enzyme dihydrofolate reductase and

thereby interferes with the generation of tetrahydrofolate, required for *de novo* synthesis of purines. However, the successful management of the drug side effects by folate supplementation without compromising its efficacy seems to contradict the above mechanism. The dominant hypothesis explaining methotrexate efficacy is based on the drug ability to increase the extracellular levels of adenosine (cAMP), and the subsequent binding of adenosine to its receptor A2A subtype results in immunosuppression through suppression of neutrophil and macrophage pro-inflammatory function, pro-inflammatory cytokine production, and inhibition of lymphocyte activation and proliferation[142,143].

If the use of methotrexate is contradicted, the prescription of alternative csDMARD such as leflunomide or sulfasalazine is preferential to any targeted DMARD, due to their oral formulation and a smaller risk of serious side effects. In case of patients failing to respond to a csDMARDs mono- or combination therapy within first 3 to 6 months, addition of biological DMARD (or targeted synthetic DMARD) is suggested as a second line treatment.[141]

1.1.7.3 Biological disease modifying anti-rheumatic drugs (bDMARDs)

1.1.7.3.1 Anti-TNF therapy

In contrast to conventional DMARDs, the biological DMARDs were developed to target specific molecules such as cytokines or cell surface receptors involved in the aberrant immune response. Among those molecules, the first cytokine validated as a therapeutic target was tumour necrosis factor (TNF), which has a central role in potentiating synovial inflammation, synovial hyperplasia, and subsequent cartilage and bone degradation in RA setting.[144,145,146,146] In line with this, on the biological level TNF inhibition results in downregulation of a range of inflammatory cytokines, diminished leukocyte recruitment to the joints, reduced angiogenesis and lower levels of matrix metalloproteinases.[147,148] Clinically, this

translates into efficient and sustained symptomatic control alongside halted radiographic joint damage.[149]

There are currently 5 anti-TNFα compounds approved for clinical use: infliximab and adalimumab (recombinant monoclonal anti-TNFα antibodies), golimumab (human anti-TNFα antibody) and etanercept (soluble TNF receptor-Fc fusion protein), and certolizumab (pegol)(humanised Fab fragment conjugated to polyethylene glycol). While all the compounds function by binding soluble form of TNFα and inhibiting its interaction with TNF receptor complex, they have different pharmacokinetic and pharmacodynamic profiles which might determine their differences in efficiency and safety.[150,151] TNF inhibitors are recommended over csDMARDs as initial treatment for the patients with moderate to severe disease activity, as they allow to achieve more rapid symptom improvement with comparable efficacy[152]. Combination therapy of any anti-TNF agent with methotrexate demonstrated superior efficacy compared to either drug used as a monotherapy, leading to a reduction in inflammation, inhibition of radiographic progression, improvement in physical function, and when using infliximab or etanercept, the achievement of clinical remission in higher proportion of patients.[153,154,155,156,157,158,159] The common adverse effects of the therapy include the occurrence of serious infections, such as tuberculosis and pneumonia, and injection site reactions, however, these are rare and usually well-tolerated. Other shortcomings include high cost and the secondary response failure (loss of response to infliximab and adalimumab) due to the formation of anti-drug antibodies.[160]

Patients failing to respond to the initial anti-TNF agent may benefit from switching to a second anti-TNF drug, with this recommendation applying to other biologic DMARDs. In the absence of response, no significant advantage was demonstrated between switching to the different agent with the same mode of action compared to a bDMARD targeting a different pathway. If the treatment target (low disease activity or remission) is reached and maintained for at least 6 months, the successful therapy is continued with potential dose reduction and increasing intervals between treatment. [142,141]

1.1.7.3.2 IL-6 receptor inhibitors

IL-6 is another major pro-inflammatory cytokine with pleiotropic function, which has an important role in the pathogenesis of RA. Elevated levels of IL-6 were detected in the serum and synovial fluid of RA patients, and significantly correlated with the disease activity and radiological joint damage.[161,162] In the synovium, IL-6 is mainly produced by resident fibroblast-like synoviocytes (FLS), and in turn acts in autocrine fashion by promoting secretion of other bioactive molecules (e.g. interferon gamma (IFNy), receptor activator of nuclear factor-κB ligand (RANKL), matrix metalloproteinase 3 (MMP-3)) ,which contribute to arthritic symptom induction, stimulating FLS proliferation, as well as inducing other immune cell activation, thereby creating a self-sustained inflammatory loop.[163] IL-6 is also responsible for systemic RA symptoms such as fatigue, anaemia and acute phase response.[164,165,166,167] All the above distinguish IL-6 as an attractive candidate for therapeutic targeting.

The IL-6 inhibitors are represented by tocilizumab (TCZ), a humanized anti-IL-6R antibody, and more recently developed sarilumab, a human anti-IL6R antibody, which has higher affinity and longer half-life but demonstrates efficacy similar to its predecessor.[168,169] TCZ functions by binding to both membrane-bound and soluble IL-6R, preventing IL-6 binding to IL-6R and thus interfering with IL-6R/gp130 receptor complex formation, necessary for IL-6 signaling.[169] TCZ is used patients with moderate to severe RA and shows favourable efficacy in DMARD-naïve patients, as well as patients refractory to csDMARD, methotrexate or TNF inhibitor treatment.[170,171,172,173] A notable benefit of TCZ is the improvement of clinical symptoms and halted joint damage when used as a monotherapy, but the addition of csDMARDs may further enhance its therapeutic effect.[174,175] Importantly, TCZ reduces fatigue and morning stiffness while also improving physical function, thus positively influencing the patient quality of life.[176] The drug also has low antigenicity, thus appears to be retained for longer than TNF inhibitors and has lower incidence of secondary response failure.[177,178] TCZ withdrawal most commonly occurs due to adverse events such as stealth

infections, presenting a serious problem due to TCZ suppressing early infection symptoms which are often overlooked. Other side effects include lower gastrointestinal tract perforations, liver enzyme abnormalities leading to liver damage, with neutropenia, thrombocytopenia, and dyslipidaemia also being common.[178]. Drug-free remission is attainable in patients both receiving TCZ long-term as a monotherapy and in combination with methotrexate, and the sustained low disease activity is achieved with the removal of concomitant administration of csDMARDs or with continued methotrexate use, respectively.[179,180]

1.1.7.3.3 Anti-IL1 receptor antagonist

IL-1 is another important pro-inflammatory mediator in RA, which promotes fibroblast-like synoviocytes to proliferate and, alongside with chondrocyte, to secrete matrix metalloproteinases, as well as indirectly promotes osteoclastogenesis, thereby potentiating cartilage degradation and bone erosion.[181] IL-1 signals by binding to biologically active IL-1R1 and engaging an accessory protein IL-1R-AcP, while the naturally occurring IL-1 receptor antagonist (IL-1Ra) competitively binds to the IL-1R1 with no subsequent signal transduction, thereby regulating the biological activity of IL-1.[181] In patients with RA, the balance between IL-1 and IL-1Ra is dramatically skewed towards IL-1 overproduction, and the elevated plasma IL-1 levels closely correlate with disease activity measures.[182,183]With that in mind, a recombinant human form of IL-1Ra, anakinra, was developed as near-identical mimic of the endogenous IL-1Ra molecule.[184] However, since the complete IL-1 signaling blockade requires a high dose of the drug, anakinra needs to be delivered by subcutaneous injection daily, which is somewhat inconvenient for the patients.[185] Anakinra was shown to achieve a significant clinical response and reduce radiographic disease progression, either alone or combined with methotrexate, but the magnitude of the improvements was inferior to TNF inhibitors.[186,187,187,187] Anakinra can nevertheless be used in patients who have medical contraindications to anti-TNF therapy, experience an insufficient response or serious adverse events with the latter.[187] The main advantage of the therapeutic IL-1Ra is a superior safety

profile with the most common adverse effect being injection site reactions, while the risk of serious infection occurrence is similar to that with TNF inhibitor treatment.[185] Similarly, patients with comorbidities such as congestive heart failure or demyelinating disease may benefit from anakinra therapy as opposed to anti-TNF compounds.[188] Overall, anakinra has good efficacy and tolerability profile but is of limited use due to the superior efficacy of other bDMARDs.

1.1.7.3.4 Anti-CD20 antibody

Therapeutic agent rituximab, developed for the treatment B cell non-Hodgkin's lymphoma, was recently adopted as another modality for RA management.[189,190] Rituximab is a chimeric antibody consisting of mouse-derived variable domain linked to the human constant domain, and recognising CD20 molecule, expressed by pre-B cells, mature naïve and memory B cells, but absent on pro-B cells, plasmablasts, and plasma cells.[191,192] By binding to CD20 on B cell surface, rituximab mediates B cell depletion by means of antibody-dependent cell-mediated cytotoxicity, complement activation and B cell apoptosis.[193,194,195] In line with B cell role in RA pathogenesis, B cell depletion results in the elimination of antigen-presenting B cells and the associated reduction in autoreactive T cell activation, reduced cytokine production and diminished levels of autoreactive antibodies and associated immune complexes.[196,197] The treatment achieves transient depletion of circulating CD20 B cells and synovial B cells to various degree, but the extent of the clinical response does not correlate with the magnitude of cell depletion.[198]

Rituximab is a therapeutic option for patients with inadequate response to TNF inhibitor treatment, with the efficacy being better for individuals failing only one anti-TNF agent rather than multiple.[199,200] In particular, rituximab is shown to achieve a superior clinical response and radiographic damage reduction in patients seropositive for RF or ACPA autoantibodies.[201,202] B cell depletion following rituximab injection is sustained on average for 6 months, and the re-

treatment is most effective when administered according to treat-to-target strategy (usually at 24 weeks) rather than upon patient experiencing symptom exacerbation (flare) [202,203] The repeated treatment is efficacious and well tolerated, and may potentially increase sensitivity to the previously inefficient therapeutic option.[204] Overall, rituximab had a favourable safety profile compared to other DMARDs, with the common adverse effects being infusion-related reactions as well as leukopenia and hypoglobulinemia, which increase the risk of serious infections.[205]

1.1.7.3.5 T-cell co-stimulation inhibition

Due to their ability to activate macrophages and synovial fibroblasts, potentiate osteoclastogenesis and bone resorption, and provide help to B cells, T lymphocytes represent the crucial players in RA pathogenesis, and therefore the modulation of their function was explored in therapeutic purposes. To become fully activated, T cells require at least two signals from the antigen-presenting cell (APC): the initial engagement of T-cell receptor with its specific antigen (presented on the APC cell surface in the context of MHC-II molecule) and the secondary, co-stimulatory signal, provided by from the interaction between CD28 and its ligand CD80/86 (B7), expressed by T cells and APCs, respectively[206]. Following activation, T lymphocytes express cytotoxic lymphocyte antigen-4 (CTLA-4) surface molecule, which exhibits 10 to 20-fold higher affinity to CD80/86 than CD28 and delivers an inhibitory signal suppressing activation of both naïve and effector T cells, thus acting as a negative regulator of T cell-dependent immune responses[207].

With that in mind, abatacept was designed as a recombinant fusion protein comprising the extracellular domain of human CTLA-4 and modified Fc portion of human IgG1. By selectively binding to CD80/86, abatacept effectively abolishes T cell activation, while also modulating the potential of other cells, including monocyte migratory capacity[208], and synovial fibroblast and B cell activation[209,210]. Abatacept is found to be clinically effective in methotrexate-naïve patients with early disease, or in patients with moderate to severe RA who

have an inadequate response to either conventional or biologic DMARDs.[211,212,213] Abatacept is shown to achieve a good clinical response and physical function improvement when administered alongside methotrexate in methotrexate-refractory patients, and this combination treatment resulted in 53% of patients achieving sustained remission[214]. The specific features of abatacept treatment include a progressive improvement in responses over time and the superior efficacy in RF- or ACPA-positive patients, as well as the greater efficacy achieved when the treatment is initiated early in the disease course (rather than following exposure to one/multiple bDMARDs)[214,215,216]. The common side effects include injection site reactions and serious infections, with the infection risk inferior when compared with other bDMARD therapy. The second generation CTLA4-Ig compound, belatacept, binds CD80/86 with higher affinity than abatacept) and thus exhibits enhanced immunosuppressive properties, but is currently only employed in transplantation[217].

Table 1-2. Conventional synthetic and biologic DMARDs currently approved for RA treatment*

Drug category and name	Structure	Mode of action	Administration route	Adverse events
Conventional synthetic DMARDs				
Methotrexate	Small molecule compounds	Competitive folate inhibitor, blocks purine synthesis	Oral	GI intolerance, stomatitis, headache, fever, skin rash, hepatic transaminase level elevation, hepatotoxicity, hypersensitivity pneumonitis severe myelosuppression (rare)
Sulfasalazine		Unknown		Gi intolerance, headache, hypersensitivity reactions (cutaneous), leukopenia, hepatic dysfunction, oligospermia
Leflunomide		Dihydroorotate dehydrogenase inhibitor, blocks *de novo* pyrimidine synthesis		GI intolerance (nausea, diarrhoea), hepatic transaminase level elevation, skin rash, alopecia, foetal/neonatal toxicity
Hydroxychloroquine		Unknown		GI intolerance (nausea, diarrhoea),rash, changes in skin pigmentation (e.g. dark spots),muscle

				weakness, retinal toxicity/retinopathy (rare)
Biologic DMARDs				
Etanercept	Soluble (TNF) receptor fusion protein	TNFα inhibitors/antagonists	Subcutaneous	Serious infections (e.g. pneumonia), reactivation of tuberculosis, opportunistic infections, non-melanoma skin cancer, exacerbation of demyelinating disease, congestive heart failure, neutropenia, injection site/infusion reactions, other cutaneous manifestations (psoriatic-like skin lesions, leukocytoclastic vasculitis)
Infliximab	Mouse/human chimeric monoclonal antibody		Intravenous	
Adalimumab	Human monoclonal antibody		Subcutaneous	
Golimumab	Human monoclonal antibody		Subcutaneous	
Certolizumab (pegol)	PEGylated Fab fragment of humanized monoclonal antibody		Subcutaneous	
Tocilizumab	Humanized monoclonal antibody	IL-6 receptor inhibitors	Intravenous	Serious and stealth infections, lower GI tract perforations (rare), liver enzyme abnormalities, neutropenia, thrombocytopenia, dyslipidaemia, injection site reactions
Sarilumab	Human monoclonal antibody		Subcutaneous	
Anakinra	Recombinant human IL-1 receptor antagonist	IL-1 receptor antagonist	Subcutaneous	Injection site reactions, serious infections (pneumonia), RA progression
Rituximab	Mouse/human chimeric monoclonal antibody	$CD20^+$ B cell depletion	Intravenous	Serious infections, injection site reactions, leukopenia, hypoglobulinemia, Hepatitis B reactivation
Abatacept	Recombinant human CTLA-4-IgG fusion protein	T-cell co-stimulation inhibition (via CD80/86 binding)	Subcutaneous	Serious infections, injection site reactions (mild/moderate)

*For summary on targeted synthetic DMARDs (JAK inhibitors) see **Table 1-3Error! Reference source not found.**.

1.1.7.3.6 Janus kinase (JAK) inhibitors

The arrival of biologic DMARDs and subsequent optimization of treatment strategies (i.e. treat-to-target approach) dramatically transformed the therapeutic outcomes for the RA patients by improving their physical function, suspending/halting disease progression and enhancing the overall quality of life. However, a substantial proportion of patients fail to achieve a therapeutic goal (low disease activity or remission) with bDMARD therapy, even after switching to the second- and third-line agents. Moreover, some patients develop the secondary therapeutic failure (i.e. loss of drug efficacy) due to immunogenicity of biologic agents and the development of drug-neutralizing antibodies. Other important limitations, which may result in bDMARD therapy discontinuation/non-adherence, include drug-related toxicity, increased risk of serious infections and malignancies, along with the requirement for drug subcutaneous/parenteral administration and associated challenges. Importantly, despite the ongoing and clinically effective treatment, the patients reported the fatigue, chronic pain, and levels of physical disability to remain a considerable and unresolved burden, thus emphasizing the additional unmet requirement for novel therapies with an alternative mode of action.

The attention was thus drawn to the protein kinases, the intracellular signalling cascade components responsible for mediating the signal transduction downstream of the cytokine receptors and thereby representing the alternative targets for therapeutically modulating cellular responses to cytokine stimuli. The advantages of agents targeting and inhibiting enzymatic activity of protein kinases included their ability to simultaneously modulate multiple intracellular signals and their low molecular mass allowing for their oral administration (in a form of a pill). The most rigorous and effective developments to date were made in targeting Janus kinases(JAKs), which are essential and non-redundant in transmitting the signals from a variety of cytokines and hormone-like growth factors and thereby mediating the resulting changes in metabolism and function of immune, hematopoietic (and synovial tissue/resident) cells. Among the JAK-mediated stimuli are the proinflammatory cytokines which are associated with

both innate and adaptive immunity and are linked to the pathogenesis of multiple autoimmune disorders, including rheumatoid arthritis. The paramount relevance of JAK kinase therapeutic targeting was acknowledged after the characterisation of human JAK3 deficiency, which phenotypically mimicked the y-chain deficiency and manifested with severe impairment limited to the immune system.[218,219] This suggested that inhibition of JAK kinases would allow the selective modulation of immune/hematopoietic system function.

The first JAK inhibitor to be clinically approved for the management of moderate-to-severe RA was tofacitinib, a potent inhibitor of JAK3 and JAK1 kinases.[220] A number of clinical trials established tofacitinib to effectively and significantly reduce the disease activity in several patient populations, including treatment naïve and methotrexate refractory patients, and, most importantly, in individuals failing multiple biologic DMARDs with distinct action mechanisms.[221,222,223,224] Strikingly, the significant clinically meaningful response rates were observed as soon as 2 weeks from treatment onset, while the drug also notably improved the patient-reported outcomes including physical function and pain.[225] The success of tofacitinib promoted the intensive development of this class of compounds, resulting in approval of baricitinib, a JAK2/JAK1 inhibitor, for the treatment of RA.[225] While these first-generation JAK inhibitors exhibited promising efficacy and acceptable safety profile, the second-generation agents recently entered the development with the aim of restricting their inhibitory capacity to a single JAK kinase and thereby attempting to further enhance their efficacy and reduce safety concerns. So far, a JAK1 inhibitor upadacitinib is the only FDA-approved second-generation compound, with several JAK1 and JAK3-specific inhibitors being at different stages of clinical testing for both RA and other common autoimmune disorders.

The following sections review the components, mechanisms, and biological relevance of JAK/STAT pathway, as well as outline a more in-depth rationale of JAK inhibitor development and their current clinical use.

1.2 JAK-STAT pathway and implications of its therapeutic targeting

1.2.1 Signalling mechanism and functional role of the JAK-STAT pathway

1.2.1.1 Overview of the pathway discovery

Cytokines and growth factors play an essential role in communication between cells, coordinating cell growth and differentiation, metabolism and haematopoiesis while also mediating host defence, immunomodulation, and autoimmunity. Some of these soluble factors, including interferons (IFN), erythropoietin, growth hormone and prolactin, were discovered over half a century ago, and are now among the many members (>50) of the Type I and II cytokine receptor superfamily, which employ Janus kinase (JAK)/Signal transduction and activator of transcription (STAT) signalling pathway. However, other cytokines, such is interleukins 1 (IL-1) and 8 (IL-8), tumour necrosis factor (TNF) and transforming growth factor β (TGFβ) utilize different receptor subgroups and alternative intracellular signalling pathways.

While the role of cytokines was shortly established to be crucial, there was a gap in understanding how exactly they mediate the resulting specific changes in the cell. Thus, the initial insights into JAK-STAT pathway emerged (in late 1980's) from the concurrent work of Stark[226] and Darnell[227] groups on identifying the key components of the IFN-dependent signalling. Following the identification of the rapidly inducible IFN-stimulated genes (ISGs) and the associated IFN-stimulated response elements (ISREs) at the promoter region, the multimeric nuclear complexes were found to bind to the ISREs, with the two of the elements (of the complex) being denoted as the first signal transduction and transcriptional activator proteins (STATs) STAT1 and STAT2[228,229,230]. One of the unique and crucial features of the STATs was phosphorylation of their tyrosine residue, suggesting their direct involvement in the signalling pathway. The subsequent studies revealed that the STAT proteins were able to directly interact with

cytokine receptors and then shuttle/translocate from the cytoplasm to the nucleus to potentially exert their transcription activator function[231].

In the meantime, several tyrosine kinases, Janus kinases (JAKs) (JAK1 and JAK2) and Tyrosine kinase 2 (Tyk2), were already identified as a new class of protein kinases, but their functional significance remained unknown[232,233,234]. c. This discovery not only placed the JAK kinases as the linking element between the cytokine receptor and the STAT protein, but also demonstrated their indispensable role in the cytokine signalling. Over the next few years, the catalogue of all existing JAK and STAT proteins was completed, and they have been linked/assigned to their corresponding cytokines. Shortly thereafter, a loss-of-function JAK3 mutation was found to underly a severe combined immunodeficiency (SCID) phenotype in a patient, thus directly demonstrating the clinical relevance of the JAK-STAT pathway[235].

1.2.1.2 Principal components: JAK and STAT proteins

JAK kinases represent one of the key members of the JAK/STAT pathway. They are tyrosine kinases, thus function by phosphorylating a specific tyrosine residue on their target molecules, namely other JAKs molecules, cytokine receptors and STAT proteins, thereby promoting their activation. The mammalian family of JAK kinases consists of 4 members-JAK1, JAK2, JAK3 and Tyk2, with JAK1, JAK2 and Tyk2 being ubiquitously expressed and JAK3 expression limited to hematopoietic cells. All members are comprised of 7 JAK homology (JH) domains which form 4 structural domains, including four-point-one, erzin, radixin, moesin (FERM) domain (JH5,6 and 7) and Src homology 2 (SH2) (JH3 and 4) domain. A unique feature of JAKs is the presence of two structurally related kinase domains- a ‘true’ kinase domain (JH1) adjacent to a catalytically inactive pseudo-kinase (JH2), with the latter domain negatively regulating the JH1 enzymatic activity[236]. This architectural duality has prompted the kinases to be named after Janus, the two-faced Roman god of doors and new beginnings. JAKs are constitutively associated with intracellular domains of distinct cytokine receptor

subunits, and that in turn determines the pairing of the kinases, necessary for their function. For instance, JAK3 only associates with common gamma chain (γc) subunit, and thus always pairs with JAK1 to convey the signals from γc cytokines IL-2, IL-4, IL-7,IL-9, IL-15, IL-21, which are critical for lymphocyte maturation, differentiation and homeostasis. JAK1, on the other hand, can associate with several different receptor subunits and, in conjunction with JAK2 and/or TYK2, mediate the signalling of gp130 family cytokines (IL-6, IL-11, oncostatin M, leukaemia inhibitory factor (LIF)), as well as Type I interferons (IFNα/β) and IFNγ. JAK2 is the only member which couples with itself and is indispensable in regulating the signalling of cytokines (IL-3,IL-5), hormone-like cytokines (erythropoietin, growth hormone, prolactin) and growth factors (granulocyte-macrophage colony-stimulating factor (GM-CSF)). All the existing JAK combinations, their associated STATs and signalling outcomes are illustrated in Figure 1-1.

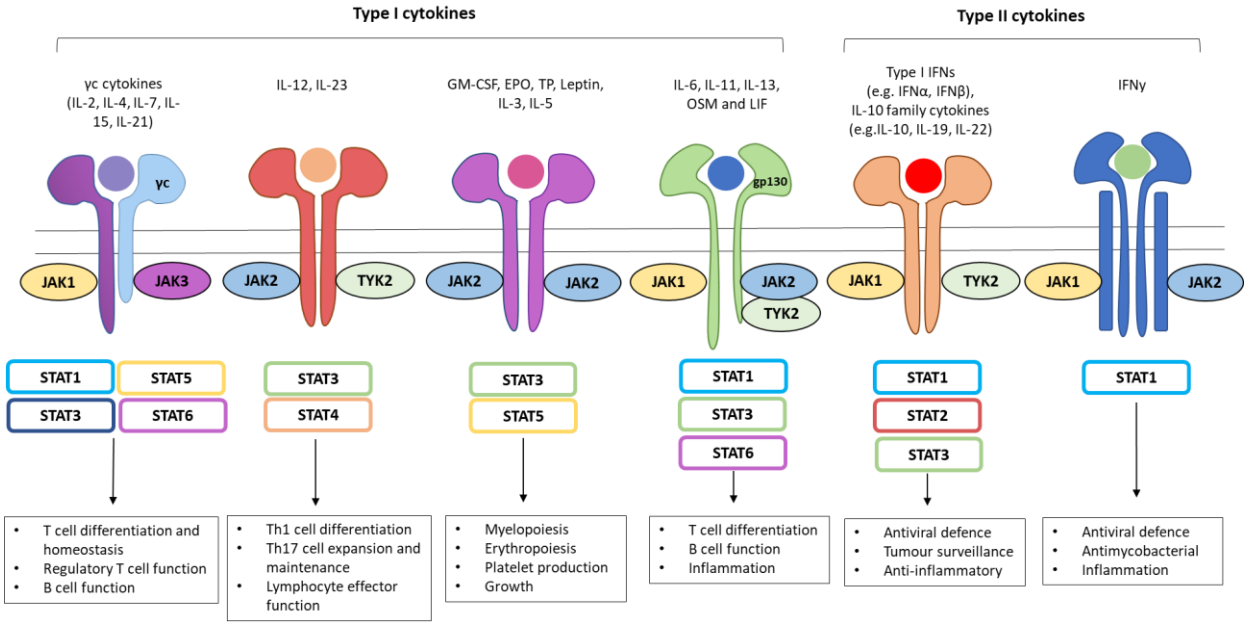


Figure 1-1. Type I and Type II cytokine receptors, their associated JAK- and STAT- family members and the biological significance of the signalling via the JAK/STAT pathway.

Adapted from [237]. Different members of JAK family selectively associate with intracellular domains of distinct cytokine receptor subunits, and that determines the combinations of kinases mediating the cytokine signalling. Distinct cytokines preferentially employ a single STAT family protein for signal transduction (e.g. Type I IFNs recruit STAT1) but can also activate other STAT members with smaller potency (e.g. STAT3 and STAT4). The ligand (cytokine) binding to the receptor promote activation and phosphorylation of JAKs, which in turn recruit and activate latent cytoplasmic STAT proteins. Activated STATs form homo- or heterodimers, which translocate into the nucleus and act as transcription factors mediating the expression of target genes, products of which are involved in a range of crucial biological functions, including hematopoietic cell differentiation and function, immune host defence, inflammation and tumour surveillance.

The signal transduction and activator of transcription (STAT) proteins are latent cytoplasmic transcription factors and have a dual role as intracellular mediators of responses to cytokine stimulation and nuclear activators of cytokine-induced genes. The mammalian STAT family is represented by 7 members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) which share a highly conserved structure. STATs are distinguished among transcription factors by 2 unique structural features- Src homology 2 (SH2) domain, which mediates their recruitment from the cytosol, interaction with activated upstream receptors and JAK kinases, as well as dimerization with other STATs, and a conserved tyrosine residue within transactivation domain, which becomes phosphorylated by JAKs and also participates in dimer formation. Other important domains include DNA-binding domain, determining the specificity for DNA binding and carrying nuclear import-export signals, and N-terminal domain, responsible for STAT tetramer formation (via dimer-dimer interactions) and their cooperative DNA binding. Latent STAT proteins, activated by the means of phosphorylation, typically carry out their function as homodimers, but can also form heterodimers (STAT1-STAT2, STAT1-STAT3, STAT-STAT4), which are thought to diversify the range of specific signals conveyed by cytokines, given the limited number of available STAT proteins[238]. In the context of canonical JAK-STAT signalling, phosphorylation and dimerization of the STAT molecules appears to be a prerequisite for their activity, enabling them to translocate from cytoplasm to the nucleus and promote the transcription of their target genes.

Following their activation, STAT dimers translocate from cytoplasm into the nucleus, where they were initially found to function as classical transcription factors by directly associating with DNA-response elements and mediating transcription of associated target genes. For instance, all STAT homodimers (except for STAT2) bind gamma interferon activation site (GAS) elements containing a palindromic DNA motif (although with slightly different affinities), whereas STAT1-STAT2 heterodimer together with IRF9 form an interferon-stimulated gene factor 3 (ISGF-3) complex which engages with ISRE enhancer (in response to Type I IFN signalling)[239,240].The advent of new-generation sequencing and its coupling with chromatin precipitation (ChiP-seq) allowed a comprehensive genome-wide mapping of the STAT-DNA binding, which, when complemented with loss- or gain-of-function transcriptomic approach, allowed

to identify genes which are both engaged by STATs and represent a target of STAT-dependent transcriptional regulation. This approach revealed STATs not only to localise at multitude of DNA-response elements other than GAS, proximal to target genes, but also to commonly associate with and instruct the distal binding sites such as enhancers, epigenetic hotspots and non-coding loci (micro-RNAs and long intergenic non-coding RNAs)[241]. Notably, STATs appeared to be crucial in permissive epigenetic remodelling of enhancers at T cell lineage-specific loci, including *Ifng, Il-4/Il-13 and Il17a/Il-17f* [242]. Moreover, specific STATs were found to act upstream of the 'master' transcription factors such as T-bet and GATA3 and contribute to the T helper cell lineage commitment by creating an activate lineage-specific enhancer epigenetic landscape, while concurrently supressing the enhancers linked to the alternative cell fate[243]. Similar STAT-dependent epigenetic remodelling also occurred in T lymphocytes and other immune cells at the gene loci essential for inflammatory and homeostatic cell functions and is mediated by the deposition of permissive or repressive epigenetic marks[244,245,246]. The recent evidence suggests STATs to re-shape the epigenetic landscape through recruitment and interaction with chromatin modifiers, such as (histone) methyltransferase EZH2, which in turn mediates H3K27me3 (tri-methylation of His3 at Lys27) deposition and thus the (target) gene silencing[246]. Genome-wide correlation of STAT DNA-binding patterns with those of the other transcription factors (TFs) identified that STATs can also combine with other TFs to form multi-molecular transcriptional regulatory networks. Thus, STAT3 in embryonic stem cells is found to cooperate with a network containing Oct4, Smad2 and NANOG TFs in shaping cell identity, while in T cells STAT3,together with IRF4 and BATF, initiate a transcriptional program which is subsequently coordinated by Th17 helper lineage-defining TF RORγt[247,248].

1.2.1.3 Canonical signalling pathway and its regulation

The JAK-STAT canonical signalling cascade is initiated by the ligand binding to the (Type I/II) cytokine receptor, leading to receptor subunit multimerization

(dimerization or heteromultimer formation). This reorientation of receptor subunits, associated with JAKs, brings two JAKs in close proximity to each other and allows their transphosphorylation (at the kinase domain JH1). In turn, activated JAKs phosphorylate tyrosine residues on the (cytokine) receptor cytoplasmic tail, creating the 'docking sites' for the recruitment and binding of latent STAT family proteins via their SH2 domains. The recruited STAT monomers are then activated by tyrosine phosphorylation at their transactivation domain by JAKs, and these phosphotyrosine residues are reciprocally recognised by SH2 domains of the partner STAT proteins, leading to 'parallel' dimer formation. The resulting STAT dimers are recognised (through their nuclear localisation signal) by importin α/ß complex and transported the nucleus, where Ran GTPase promotes the dissociation of STAT from the complex. In the nucleus, STATs engage with the promoters of the target genes and mediate their transcriptional activation or repression, alongside with exhibiting other DNA-associated functions described above. The STAT activity is transient (in the normal cells), and upon dephosphorylation in the nucleus inactivated STATs are transported back to the cytoplasm and utilized during the next signalling event, thereby completing the activation/inactivation loop. Thus, the JAK/STAT pathway has a remarkably uncomplicated design, facilitating an immediate conversion of extracellular cues into the transcriptional response. However, the seemingly straightforward JAK-STAT paradigm also entails several layers of complexity, which involve the regulation of signalling and the non-canonical aspects of the pathway, discussed further.

Considering that the molecular products of STAT-dependent genes are involved in mediating proliferation, differentiation and survival of both hematopoietic and immune cells, the aberrant activation of any of the cascade components may result in the development of immune disorders and hematologic malignancies. Thus, the amplitude and kinetics of the JAK-STAT signalling are tightly regulated at the multiple levels of the pathway and involve several distinct mechanisms.

The main regulatory components are a range of protein tyrosine phosphatases (PTPs), proteins of PIAS (protein inhibitor of activated STAT) family as well as suppressor of cytokine signalling (SOCS) proteins. Cytoplasmic tyrosine

phosphatases, such as SHP1 and SHP2, employ their SH2 domain to interact with a phosphotyrosine on activated cytokine receptors and JAK kinases, thereby promoting their dephosphorylation and preventing further activation of the downstream STATs.[249,250] A transmembrane PTP CD45, expressed by all hematopoietic cells and crucial in mediating antigen receptor signalling in T and B lymphocytes, also acts as a negative regulator by associating with and directly inactivating/dephosphorylating JAK kinases[251]. The regulatory modules can also directly target phosphorylated STAT dimers both in the nucleus and the cytoplasm. The most prominent STAT tyrosine phosphatase is a T-cell PTP (TCPTP), found in both cytoplasmic and nuclear compartments and known to preferentially mediate dephosphorylation of STAT1, STAT3 and possibly STAT5[252]. Since a 'parallel' STAT dimer conformation, established by reciprocal binding of partner protein SH2 domains to phosphotyrosines, has a concealed tyrosine residue, the phosphatases target the inverted, 'anti-parallel' dimers, formed by STAT N-terminal interactions and thus bearing phosphotyrosine at the more accessible site[252].

Protein inhibitor of activated STAT (PIAS) are constitutively expressed nuclear proteins, represented by 4 family members (PIAS1, PIAS3, PIASX and PIASy) and implicated in the negative regulation of activated STAT dimers. Thus, PIAS3 is known to specifically interact with STAT3 and inhibit its DNA-binding capacity and associated gene activation[253]. On the other hand, PIASy exhibits an adaptor protein function, recruiting other co-repressors and thereby blocking STAT1-dependent transcription[254]. In addition, PIAS were recently described to exhibit E3-type ligase function and conjugate ubiquitin-like SUMO molecules to STAT1 (a process termed 'sumoylation), which in turn inhibited STAT1 transcriptional activity[255].

Suppressor of cytokine signalling (SOCS) family proteins are the third and most prominent facet of JAK-STAT signalling attenuation. SOCS proteins operate through a 'classic' negative feedback loop: their expression is cytokine-induced and mediated by activated STAT proteins, and the SOCS proteins in turn downregulate the pathway by interacting with activated receptors, kinases, and STATs . The 4 members of the family (SOCS1, SOCS2, SOCS3 and cytokine-inducible SH2-domain protein (CIS)), acting as the negative regulators, all

contain SH2 domain for binding to the phosphorylated tyrosine residues as well as adjacent SOCS box domain, but each employ distinct inhibitory mechanisms[256]. Thus, SOCS1 via SH2 domain binds phosphotyrosine residue within JAK activation loop, thereby directly blocking its kinase/enzymatic activity.[257] SOCS3 protein can both interact with JAKs or bind to the activated cytokine receptor phosphotyrosine proximal to JAKs and subsequently inhibit their function.[258] Meanwhile, CIS proteins appear to interfere with STAT activation by competing with STAT monomers for the binding to the (activated) cytokine receptor 'docking' sites. Additionally, SOCS proteins can employ their SOCS box domain to interact with the elongins B and C, components of the ubiquitin E3 ligase complex, indicating their possible role in targeting proteins for proteasome-mediated degradation.[259] Apart from controlling the duration and magnitude of the (JAK-STAT) pathway activity, SOCS proteins appear to fine-tune the quality/specificity of cytokine responses, as exemplified by the shift from the IL-6 induced gene expression profile towards IFNy-induced transcriptional program in response to IL-6 in the absence of SOCS3. Through this functional ability, SOCS proteins mediate a range of (cytokine-dependent) crucial processes in the immune cells, including early T lymphocyte development and determination of cell fate during Th cell differentiation, and play a key role in regulatory T cell function and macrophage polarization.[259,260]

In addition to the above canonical aspects of the superficially simple JAK-STAT pathway, its signalling is further refined by the intricate modifications mostly involving STAT proteins, and the additional dimension of complexity arises from the pathway interaction with other signalling cascades. These non-canonical elements of JAK-STAT signalling are briefly outlined in the next section.

1.2.1.4 Non-canonical aspects of JAK-STAT pathway and crosstalk with other signalling cascades

In contrast to the activated STAT dimers operating in the canonical JAK-STAT cascade and entering the nucleus exclusively in phosphorylated form, STAT1 and

STAT3 proteins are also detected in the nucleus in the absence of tyrosine phosphorylation. These unphosphorylated STATs (U-STATs) also utilize a distinct nuclear import strategy, surpassing the need for carrier proteins and directly interacting with nuclear core proteins, which likely allows them to shuttle between cytoplasmic and nuclear compartments[261]. In the nucleus, U-STAT3 proteins act as transcription factors predominantly mediating the expression of genes not targeted by their phosphorylated counterparts, including several oncogenes[262]. U-STAT1, on the other hand, can enhance the STAT1-dependent gene transcription and thereby promote sustained anti-viral and immune responses[263]. Thus, U-STATs act as secondary transcription factors in scenarios where the expression of the biologically active molecule, initially mediated by transiently phosphorylated STATs, is most advantageous for the cell response if sustained long-term[261]. This capacity is facilitated by the increase in U-STAT levels following a phosphorylated STAT-dependent expression of the corresponding STAT gene, which persist long-term after phosphorylated STAT deactivation[261]. In addition, U-STAT3 is involved in a crosstalk with nuclear factor Kappa B (NFκB) pathway, whereby accumulating in response to IL-6 stimulation and cooperating with U-NFκB it promotes the expression of κB-dependent genes, including RANTES and IL-6, which do not directly respond to phosphorylated STAT3[264].

While the conventional signalling paradigm primarily relies on JAK kinase activity for STAT activation, STATs were also found to be tyrosine phosphorylated by a number of other molecules. The examples include receptor tyrosine kinase Flt3, promoting JAK-independent tyrosine phosphorylation of STAT5, which in turn regulates hematopoietic progenitor cell proliferation, and nucleic acid sensor STING, mediating the activation of STAT6 and thereby contributing to the anti-viral immune response[262,265]. Aside from acquiring phoshphotyrosine residue following cytokine stimulation, STAT proteins can undergo additional post-translational modifications, including serine phosphorylation, methylation, acetylation and sumoylation. All STAT proteins are found to carry at least one phosphorylated serine residue, which can be present independently of tyrosine phosphorylation, and its phosphorylation is mediated by serine kinases belonging to several signalling pathways, including ERK, JNK and p38MAPK pathways[266]. The requirement of STAT serine phosphorylation for their maximal

transcriptional activity appears to be cell-type dependent and is shown to be indispensable for transcriptional STAT1 and STAT3 responses to respective IFNγ and gp130 cytokines (e.g.IL-6) *in vitro*, as well as for hematopoietic transformation and postnatal survival in mouse models[267,268,269]. The other potential STAT activities affected by the presence of phosphoserine are thought to be DNA binding and association with other proteins, although the biological significance of those remains to be elucidated[266]. STAT acetylation, similarly to serine phosphorylation, is induced by cytokine stimulus and appears to enhance STAT functions, including transcriptional activation capacity, protein-protein interactions and dimerization[270]. On the other hand, sumoylation is considered as a primarily negative regulatory mechanism, mediating inhibition of STAT activity by directly diminishing their phoshphorylation while also indirectly promoting dephosphorylation[271].

Other factors capable of influencing the JAK-STAT signalling are the crosstalk among the JAK-STAT pathways as well as interaction with distinct signalling cascades. One of the staple examples of the JAK-STAT pathway interplay is that of Type I (IFNα/β) and Type II interferon (IFNγ) signalling, whereby the cell stimulation with (low-level/subthreshold) IFNγ triggers the enhanced STAT1 expression. In turn, STAT1 can form a positive feedback circle and sensitize/prime the cell for subsequent IFNγ exposure, while also (pre-) conditioning the cell for the increased IFNα response[272,240]. (Interestingly, another line of evidence suggests the crosstalk between interferons also happening in the opposite direction, with Type I interferons (IFNα/β) being the initial sensitizing factor[273].) JAK-STAT pathway can also act in concert with other signalling cascades such as TGF-β-induced SMAD and LPS/NFκB cascades. Thus, in neuronal progenitors the synergistic signalling of leukaemia inhibitory factor (LIF) and bone morphogenic protein-2 (BMP-2) relies on cooperative activity of respective STAT3 and Smad1 molecules for mediating their differentiation into astrocytes[274]. However, in T lymphocytes TGF-β was shown to rather shown to inhibit IL-12 signal transduction by interfering with JAK2/TYK2 and associated (downstream) STAT3 and STAT4 phoshphorylation, thereby reducing cell proliferation and IFNγ production[275].

JAK-STAT signalling cascade is a distinctively uncomplicated pathway that involves only a few key 'players' and employs a number of additional modifying mechanisms, but is nevertheless capable of mediating, in a non-redundant fashion, a remarkably diverse range of responses, in particular in immune cells, ranging from cell development and maintenance of homeostasis to immune defence and protection from cancer. Such combination of simple pathway design and the complexity of the functional role, however, can present a serious disadvantage if the signalling becomes dysregulated, since the resulting aberrations in the immune responses are extremely harmful and manifest through a variety of autoimmune disorders. Additionally, the dependency of the central pathway modules (JAKs, STATs and SOCSs) on the activation by the immediate upstream component of the cascade, along with the pathway forming an auto-regulatory negative feedback loop, implies that the impairment of any single molecule would disrupt the entire pathway and subsequently result in the development of a systemic disorder. The next section highlights the most common mutations in the JAK-STAT pathway in the context of both autoimmune disease and malignancy settings and links them with to the rationale for the therapeutic targeting of the pathway.

1.2.2 JAK inhibitor development and clinical applications

1.2.2.1 Genetic links between JAK-STAT pathway and human disorders

The utilization of the mutagenic cells lines and genetically modified (knockout and knock-in) mouse models were instrumental for delineating the association between distinct Type I and Type II cytokines and the specific JAK-STAT pathway components they recruit for signal transduction, while the resulting knockout or knock-in mouse phenotypes have further confirmed to indispensible roles of JAKs and STATs in the immune/hematopoietic cell development and immune responses. However, it was the identification of the genetic aberrations of the JAK-STAT pathway components in humans and the associated range of immune

and myeloproliferative disorders that evidenced the *in vivo* relevance of the pathway and subsequently prompted its therapeutic targeting.

A large body of evidence suggests a strong link between constitutive JAK-STAT signalling activity and oncogenesis. The initial observations revealed a multitude of primary cancers to exhibit persistent phosphorylation of STAT proteins (mainly STAT1, 3 and 5), which was mediated by dysregulation of STAT-activating kinases or abrogated negative regulator function, and contributed to oncogenic cell transformation by transcriptional activation of cardinal anti-apoptotic genes and proliferation-associated proteins[276]. Currently, a number of (somatic) gain-of-function mutations affecting JAK tyrosine kinases have been identified as causative factors of various myeloproliferative neoplasms. The most well studied is the activating JAK2 mutation V617F, located within the 'pseudo-kinase' domain (JH2), which normally confers negative regulation of the active ('true') kinase domain (JH1). JAK2 kinase is invariably utilized for signal transduction by receptors for erythropoietin, thrombopoietin and GM-CSF, which mediate production of erythrocytes and platelets/megakaryocytes from pluripotent stem cells in the bone marrow. The myeloproliferative disorders polycythemia vera (PV), essential thrombocytopenia (ET) and primary myelofibrosis (PMF) exhibit distinct clinical phenotypes but share a feature of an excessive erythrocyte and platelet production. This common clinical characteristic appears to be conferred by a V617F mutation, which is present in all PV cases and in the majority of ET and PMF patients[276,277]. Importantly, the discovery of this activating JAK2 mutation prompted the development of the novel pharmacological agent for targeted JAK kinase inhibition to treat myeloproliferative neoplasms, which subsequently inspired the development of other related compounds, termed JAK inhibitors (discussed in the next section). Other gain-of-function mutations of JAK2 are implicated in the development of acute pre-T or B-cell acute lymphoblastic leukaemia and T cell lymphoma, among other malignancies, while JAK3 kinase mutations are occasionally detected in patients with acute megakaryoblastic leukaemia (AML)[278].

Both loss- and gain-of-function mutations in JAK- and STAT-encoding genes are also associated with abnormalities in immune function, manifested as immunodeficiencies, autoimmune disorders and susceptibility to infections. An

outstanding example of such association is a link between an X-linked severe combined immunodeficiency (X-SCID) syndrome and inactivating JAK3 mutation[218,219]. Traditionally, X-SCID is known to be caused by mutations in the common cytokine receptor γ chain (also known as interleukin-2 receptor γ chain), which is an essential subunit of receptor complexes for IL-2, IL-4, IL-7, IL-9,IL-15 and IL-21 cytokines responsible for lymphocyte proliferation, differentiation and survival. As a result, X-SCID patients exhibit the dramatically reduced numbers of circulating T and NK cells along with functionally defective B cells, which makes them extremely susceptible to serious and persistent infections[279]. Based on the observations of JAK3 kinase exclusively mediating common γ chain-dependent signalling, it was subsequently discovered that inactivating mutations in JAK3 can also account for SCID with the phenotype closely resembling common γ chain deficiency[218,219]. This finding was the first to demonstrate the essential role of JAK3 in lymphoid development in humans, and subsequently lead to a proposition that the compounds developed to specifically target JAK kinases (in particularly JAK3) might have a predominantly immunosupressive mode of action, which would be especially desirable in both autoimmunity and transplantation settings.

STAT proteins are also found to be affected by loss- and gain-of-function mutations, resulting in dysregulated immune responses. A dominant negative mutation in STAT3 manifests in hyper IgG syndrome (also termed Job's syndrome), characterised by recurrent cutaneous and pulmonary infections, dermatitis, highly elevated serum IgG levels and skeletal abnormalities[280,281]. The development of such multisystem disease phenotype is explained by STAT3 involvement in signal transduction of at least six classes of receptors, while the immunological defects partially stem from the diminished STAT-3 mediated differentiation of Th17 lymphocytes and IL-17 production by other cells, necessary for neutrophil recruitment and control of fungal infections[282,283,284]. On the other hand, activating STAT3 mutations are associated with early onset multiorgan autoimmunity and lymphoproliferative disorders[285]. STAT1 deficiencies occur in a spectrum ranging from negative dominant (partial) to autosomal recessive (complete), with the former causing susceptibility to a life-threatening combination of both intramacrophagic bacteria (mycobacteria) and viruses due to abrogated IFN and IFN/-mediated signalling/responses,

respectively, while the latter selectively confers predisposition to milder mycobacterial disease while preserving anti-viral immunity[285]. Interestingly, while STAT1 activating mutation expectedly leads to the development of autoimmunity, it is also associated with chronic mucocutaneous candidiasis, potentially due to increased STAT1 activity overriding/diverting the (cytokine) signals otherwise mediated by STAT3 and driving anti-fungal responses[282]. STAT5B mediates the signalling of both IL-2 cytokine, prerequisite for regulating the differentiation and homeostasis of both pro- and anti-inflammatory T cells, and the growth hormone, therefore the patients with STAT5B deficiency exhibit a complex phenotype of autoimmunity, immunodeficiency and growth retardation[286,287,288].

In addition to the above rare monogeneic diseases, genome-wide association studies (GWAS) have also linked a multitude of more common disorders to the single nucleotide polymorphisms (SNPs) in the genes encoding Type I/II cytokines and associated JAKs and STATs. Multiple genes in the IL-23 signalling pathway are strongly implicated in the autoimmune disease development, with polymorphisms in JAK2 and STAT3 in particular being predisposing factors for Crohn's disease, Bechet's disease, psoriasis and ankylosing spondylitis, among others[289,290,291]. Polymorphisms in STAT4, which mediates signalling downstream of both IL-12 and Type I IFNs, appear to increase the risk for rheumatoid arthritis, systemic lupus erythematosus (SLE) and Sjogren's syndrome, while SNPs in STAT6, required for IL-4 signalling, have a role in asthma and allergies[292,293,294].

Altogether, this vast body of evidence, obtained through identifying the genetic links between JAK-STAT pathway aberrations and human disorder development, explicitly/unequivocally demonstrates the importance of the cytokine signalling via the JAK-STAT pathway for the normal haematopoiesis and immune function. Consequently, JAK-STAT pathway became an attractive target, since its therapeutic manipulation could become an exciting novel approach for interfering with the cytokine signalling downstream of the receptor and thereby complementing (or possibly outperforming) the existing biological therapies in management of multiple autoimmune conditions.

1.2.2.2 JAK inhibitors and their clinical applications

While the therapeutic inhibition of the JAK-STAT pathway signalling was envisioned since the identification of the fundamental JAK mutations (X-SCID associated JAK3 loss-of-function and JAK2 activating mutation V617F), it became an achievable prospect following the ground-breaking success of the targeted tyrosine kinase inhibitors, such as imatinib, in management of haematologic malignancies[219,295,296,297]. Since tyrosine protein kinase enzymes require binding of the triphosphate nucleotide (e.g. adenosine triphosphate, ATP) for their catalytic activity, the first inhibitors were designed to reversibly/competitively bind to the ATP binding site on the enzyme[298]. However, considering that the tyrosine protein kinase family, which includes JAKs, comprises of approximately 90 members with highly conserved catalytic domain, along with the notion that among JAKs themselves the sequence within the ATP binding site is almost identical, the development/discovery of the selective inhibitors represent a significant challenge. Nevertheless, the identification of the crystal structures of JAK family members and the additional targeting of the specific amino acid residues prompted the generation of inhibitors with relatively high levels of selectivity. As subsequently identified, however, the selectivity of JAK inhibitors is concentration-dependent, and at higher concentrations they can function as pan-inhibitors[298,299].

The discovery of JAK inhibitors (JAKinibs) was also in part prompted by the success in the autoimmune disease management of the targeted biologic DMARDs (such as TNF and IL-6), also designed to interfere with cytokine signalling. However, with the large proportion of the patients failing to respond to one or multiple biological agents along with the parenteral administration mode and excessive costs of these drugs, the need for the novel therapeutic modality in the autoimmunity setting was apparent[300]. Thus, the emergence of the orally administered small molecule inhibitors with comparable, if not superior, clinical efficacy, which was achieved even in patients failing to respond to multiple targeted biologics, has greatly enriched the armamentarium of the existing immunomodulatory treatment options. The following sections review the currently available JAK inhibitor specifications and clinical use, as well as the

second-generation compounds with enhanced selectivity currently under development.

1.2.2.2.1 Tofacitinib

Tofacitinib (Xelanjanz®; CP-690,55 during development) is a small molecule targeted synthetic DMARD, and the first-in-class JAK inhibitor approved for the treatment of rheumatoid arthritis. It was developed by Pfizer for the management of inflammatory conditions and was the first compound of its class to be tested in clinic. Tofacitinib was approved for the use in US by the Food and Drug Administration (FDA) for the treatment of moderate to severe rheumatoid arthritis in 2012, with the subsequent approval by European Medicines Agency (EMA) in 2017[220,301]. The originally established tofacitinib potency for selective JAK3 inhibition suggested that the compound might preferentially target JAK3-expressing lymphoid cells, thereby providing a therapeutic immunomodulation/immunosuppression effect while exhibiting limited non-immunological and haematologic cytotoxicity[302]. However, tofacitinib also appeared to potently inhibit JAK1 and to lesser extent JAK2, while having limited affinity to TYK2[302,303]. While this broader inhibitory profile underlies some of the compound adverse effects, it might also potentiate a remarkable (clinical) efficacy achieved by tofacitinib. Notably, the unique chemical structure of tofacitinib ensures its limited interference with the activity of kinases other than JAKs[304].

In vitro studies have demonstrated tofacitinib to potently block the JAK3-mediated signalling of the common γ chain cytokines (IL-2, IL-4, IL-7, IL-9,IL-15,IL-21), as well as signal transduction by JAK1/JAK2-dependent IFNy and IL-6 and to a lesser degree by JAK2/JAK2-mediated IL-12 and IL-23. Such effect on the cytokine-induced signal transduction by JAK-STAT pathway in murine $CD4^+$ T cells translated into abrogation of naïve lymphocyte differentiation into Th2 and Th17 helper subsets, as well as diminished generation of Th1 cells[305]. This result was consistent with the reduced Th1-associated IFNy and Th17-associated IL-17

production by $CD4^+$ T lymphocytes from individuals with RA[306]. Along with the significant impact of the inhibitor on naïve B cell differentiation and function and the impaired ability of the compound-treated dendritic cells for $CD4^+$ T cell activation/priming, the above observations highlight the tofacitinib potential to interfere with adaptive immune responses[307,308,309]. Importantly, tofacitinib was also demonstrated to inhibit innate immune mechanisms in the IFNy and STAT1-dependent LPS-induced sepsis mouse model, indicating the effects of the inhibitor to be similarly facilitated through modulation of innate immunity[305]. Fascinatingly, osteoclast capacity for bone resorption was also suppressed following drug exposure, thus linking the immunological mechanisms of action of tofacitinib to its ability for limiting joint damage in both mouse models and RA patients[310]. In parallel, the evaluation of tofacitinib efficacy in multiple mouse and rat models of arthritis demonstrated a rapid and significant reduction in plasma levels of inflammatory mediators, diminished joint tissue inflammatory cell infiltration alongside with dose-dependent improvement in disease clinical score and amelioration of cartilage and bone destruction[305,311,312,313]. These encouraging findings in preclinical models have facilitated the entry of tofacitinib into clinical trials for RA.

The Phase II clinical trials confirmed the safety and tolerability profile of tofacitinib, as well as its efficacy in patients with active RA in comparison to placebo or in combination with methotrexate in patients with inadequate response to methotrexate[314,315]. A further comprehensive assessement of tofacitinib efficacy was conducted in range of Phase III trials, which (together) encompassed more than 6000 patients across nearly all patient populations-from treatment-naïve to non-responders to targeted biologic DMARDs (tbDMARDs). In ORAL START trial tofacitinib was shown to be efficacious as a monotherapy in treatment-naïve patients and was superior to methotrexate at reducing disease activity and halting radiographic damage, which was not previously achieved with other available therapies. Notably, the drug demonstrated rapid mode of action, with the first improvements in disease score detected as soon as two weeks after treatment initiation[221]. ORAL Scan trial has demonstrated tofacitinib to reach primary endpoints and improve quality of life as a monotherapy in patients failing conventional synthetic DMARD therapy. Importantly, the response to tofacitinib monotherapy appeared to be comparable in subject with

both early and established disease.[222] Other trials have shown tofacitinib to be also effective in combination with methotrexate, and in this combination exhibit efficacy non-inferior to TNF inhibitor adalimumab in patients refractory to csDMARDs[316]. Most strikingly, the tofacitinib treatment achieved good clinical response in patients with previous inadequate response to one or several biologic DMARDs (TNF inhibitors)[223, 224,317,]. Across the trials, tofacitinib inhibited radiographic (joint) damage progression as well as improved patient quality of life. The two recently released long-term studies reported sustained improvement in clinical scores along with stability of (long-term) safety profile[224,318]. Supported by the above data, tofacitinib is currently approved for the management of moderate to severe active RA in patients refractory or intolerant to one or more csDMARDs. EULAR treatment recommendations were recently updated to include tofacitinib as an alternative to biological DMARDs, which can be added to the csDMARD in patients failing to meet an initial six-month target (low disease activity or remission)[141].

Due to the contribution of Type I/II cytokine signalling to the development of autoimmune conditions other than RA, the clinical trials for tofacitinib extended across the spectrum of disorders. The encouraging results were so far observed in the patients with psoriasis, psoriatic arthritis, inflammatory bowel disease (IBD) and ankylosing spondylitis[319,320,321,322]. When evaluated in *de novo* kidney transplant patients, tofacitinib was comparable to cyclosporin in preventing acute renal allograft rejection and had beneficial effect on allograft preservation and function[323]. Recently, tofacitinib was approved for the treatment of moderate to severe ulcerative colitis in patients refractory to TNF inhibitor therapy[324].

The adverse events associated with tofacitinib treatment are generally comparable to those of biologics. Opportunistic and severe infections are among most common complications reported, including *Mycobacterium tuberculosis*, *Cytomegalovirus, and Pneumocystis jirovecii* pneumonia. The absolute risk of these infections, however, is not significantly greater when compared to the biologic DMARDS, while the concurrent use of biologics, glucocorticosteroids and the development of lymphopenia greatly increase the susceptibility to infections[325,326]. The one exception is *Herpes zoster* infection, occurring at

higher rates with tofacitinib than with placebo or other DMARD therapies, but it appears to only affect certain populations and is mostly limited to its mild form[327]. The changes in lipid profile composition are observed in RA patients treated with tofacitinib, with significantly elevated levels of low- and high-density lipoproteins, but it remains unclear whether these changes can be directly linked to the cardiovascular events. Due to tofacitinib blocking signalling of interferons, essential for cancer immunosurveillance coordination, the compound use might increase the risk of malignancy development[328]. The integrated data from tofacitinib clinical trials suggests the most common cancers to be lung, breast and lymphoproliferative neoplasms along with non-melanoma skin cancers, but their incidence was within the expected range for moderate-to-severe RA patients group and did not exceed that of biologic DMARDs[329]. Other common complications associated with tofacitinib administration include headaches, diarrhoea, urinary and upper respiratory tract infections and mild neutropenia[325].

1.2.2.2.2 Ruxolitinib and baricitinib

The development of another first generation JAKinib, ruxolitinib, was prompted by the discovery of association between a JAK2 activating mutation V617F and the occurrence of several myeloproliferative disorders[295,296]. Ruxolitinib (Jakafi®) is JAK2/JAK1 inhibitor and the very first of this class to be approved by FDA in 2011[330]. It is currently licensed for the management of intermediate- to high-risk myelofibrosis, with the patients demonstrating improvement in splenomegaly, disease-associated constitutional symptoms and overall survival with only modest toxicity[331]. Ruxolitinib was also recently approved for the treatment of (uncontrolled) polycythemia vera, another myeloproliferative neoplastic disorder, in patients who are intolerant or refractory to (conventional) hydroxyurea therapy, as well as for the management of steroid-refractory graft-versus host disease[332,333]. Lastly, the topical form of ruxolitinib shows efficacy in treatment of (plaque) psoriasis, vitiligo and alopecia areata, with clinical studies currently underway[334,335,336].

Baricitinib (Olumiant®) is a structural analogue of ruxolitinib which inhibits JAK2 and JAK1 with high selectivity while largely sparing JAK3 activity. However, since JAK3 exclusively pairs with JAK1 kinase and thus mediates transduction of the same cytokine signals, the mechanistic advantage in terms of signal transduction inhibition is yet unclear[299]. Clinically, baricitinib was tested for the management of moderate to severe active RA and appeared to be the first JAK inhibitor to show efficacy with a once-daily oral dosing (4 mg)[337]. In RA-BEGIN clinical trial, baricitinib monotherapy outperformed methotrexate in efficacy in treatment-naïve patients, and in combination with methotrexate baricitinib showed superior efficacy to methotrexate alone[338]. In RA-BUILD study baricitinib treatment demonstrated clinical improvement and cessation of radiographic damage progression in patients refractory or intolerant to csDMARDs[339]. While the drug was similar to tofacitinib in achieving significant clinical improvement in patients with inadequate response or intolerance to one or more biologic DMARDs, including TNF inhibitors, baricitinib showed an unparalleled superior efficacy to TNF inhibitor adalimumab, which has not yet been achieved with other therapeutic modalities[340,341]. Baricitinib adverse event profile is largely similar to that of tofacitinib and included severe infections such as *Herpes zoster*, malignancies, thrombosis, urinary tract infections, hyperlipidaemia, and neutropenia. The outstanding efficacy and good tolerability prompted the approval of baricitinib (as monotherapy or combined with methotrexate) by FDA and EMA for the management of moderate to severe active RA in subjects refractory or intolerant to treatment by one or more biologic DMARDs[342]. The clinical trials evaluating the efficacy of baricitinib and moderate-to-severe atopic dermatitis are ongoing, while those for psoriasis and psoriatic arthritis were discontinued due to reallocation of resources for other program development.

1.2.2.2.3 Second-generation JAK inhibitors

Following the success of first-generation JAK inhibitors of relatively broad specificity, the next objective was to generate compounds with enhanced

affinity/selectivity for specific JAKs. This strategy was presumed to reduce the adverse event occurrence while retaining the favourable clinical efficacy[330]. Several JAK1-selective inhibitors were developed based on the notion of JAK1 having a dominant role over JAK3 in c chain cytokine signalling *in vitro*, along with JAK1 heterodimer signalling inhibition demonstrated to underlie the therapeutic efficacy of tofacitinib in mouse collagen-induced arthritis (CIA) [343,344]. Upadacitinib is a selective JAK1 inhibitor, with its selectivity (over other JAKs) determined by its ability to bind JAK1 at two distinct sites. Filgotinib is another JAK1 inhibitor, which forms an active metabolite with similar JAK1-selective inhibitor properties which contributes to the overall pharmacodynamic profile by prolonging JAK1 inhibition[345]. Both compounds were shown to exhibit rapid and notable dose-dependent improvement in signs and symptoms of disease when administered to RA patients refractory or intolerant to methotrexate, either alone or in combination with methotrexate, along with the tolerable safety profile[346,347,348]. In addition, upadacitinib also demonstrated rapid clinical improvements in patients with inadequate response to anti-TNF therapy[349]. Recently, upadacitinib was approved by the FDA for the management of moderate to severe RA in patients refractory or intolerant to methotrexate therapy. Other emerging second-generation compounds, peficitinib and decernotinib, are JAK3 selective inhibitors, with peficitinib exhibiting moderate selectivity and inhibiting all other JAK members, while decernotinib having a more potent JAK3 affinity[350,351]. These inhibitors have shown an efficacy and safety profile comparable to the JAK3/JAK1 inhibitor tofacitinib. While the clinical trial results employing more selective JAK inhibitors are very encouraging, further studies are required for potentially further improving their benefit/risk ratio (e.g. by dosage adjustments) and assessing their long-term efficacy and safety potential.

The full list of JAK inhibitors, both currently approved for clinical use and tested in clinical trials is presented in Table 1-3.

Table 1-3. Janus kinase inhibitors currently clinically employed or tested for the treatment of RA and other autoimmune conditions.

Compound	Molecular targets	Indication/disease	Development phase
First-generation inhibitors			
Tofacitinib	JAK3 > JAK1 ≥ JAK2	RA Ulcerative colitis Psoriatic arthritis	*FDA approved*
		Ankylosing spondylitis Juvenile idiopathic arthritis	Phase III
		Transplant rejection	Phase IIb (completed)
Ruxolitinib	JAK2,JAK1 ≥ TYK2	Myelofibrosis Polycythemia vera Acute graft-versus-host disease	*FDA approved*
		RA Psoriasis (topical) Alopecia areata, atopic dermatitis	Phase II
Oclacitinib	JAK1	Canine atopic dermatitis	*FDA approved*
Baricitinib	JAK2, JAK1 ≥ TYK2	RA	*FDA approved*
		Atopic dermatitis	Preregistration phase
		Chronic graft-versus-host disease	Phase II
		Psoriasis, psoriatic arthritis	Discontinued
Second-generation inhibitors*			
Upadacitinib	JAK1	RA	*FDA approved*
		Psoriatic arthritis, atopic dermatitis Ulcerative colitis	Phase III
Filgotinib	JAK1	RA	Submitted for FDA approval (December 2019)
		Ulcerative colitis, Crohn's disease	Phase III
		Psoriatic arthritis	Phase II
Peficitinib	JAK3	RA	Phase III/Approved in Japan
		Psoriasis, ulcerative colitis	Phase III
Decernotinib	JAK3	RA	Discontinued
Itacitinib	JAK1	RA	Phase II (discontinued)
		Graft-versus-host disease	Phase III (failed)

*The new-generation JAK-inhibitors not tested for RA are excluded

1.3 Dendritic cell-CD4$^+$ T cell crosstalk

1.3.1 The role of dendritic cells during naïve CD4$^+$ T cell activation and polarization

Dendritic cells (DCs) are bone marrow-derived 'professional' antigen-presenting cells (APCs) which are found in virtually all tissues of the body apart from brain[352]. They function as the 'guardians' of the immune system by performing surveillance of their local tissue sites for the presence of foreign/exogenous or self-antigens, and as the essential orchestrators of the immune responses through the initiation of adaptive (immune) response or induction of immune tolerance.[353] The immature DCs primarily specialise in antigen uptake and processing and are triggered to undergo activation/maturation following an intrinsic stimulus (e.g. internal milieu disturbance), or due to recognition of either conserved pathogen-associated molecular patterns (PAMPs) or damage-associated molecules (DAMPs) of endogenous origin[354,355]. During activation/maturation DCs switch from phagocytosing to antigen-presenting phenotype by upregulating the expression of MHC-II-peptide complexes and co-stimulatory molecules as well as producing high levels of inflammatory cytokines[356]. Concurrently, activated DCs enhance their motility and upregulate chemokine receptor CCR7, which allows them to migrate, via the lymphatic circulation, to the lymph node paracortex, mostly consisting of T cells[357,358]. While the expression of MHC-II and co-stimulatory molecules is also exhibited by other APCs, the above capacity of DCs to deliver the antigens from the periphery to the T cell zone in the lymph node and to concurrently provide cytokines promoting T cell differentiation into specific helper subsets makes them uniquely equipped for naïve T cell priming.

Following their maturation in the thymus, naïve T cells continuously recirculate between secondary lymphoid organs via blood or lymphatic vasculature in pursuit of (their cognate) antigen encounter[359]. Since the naïve T cells bearing/expressing a T-cell receptor (TCR) specific for a certain antigen are

present at very low frequencies, their initial frequent transient interactions with (antigen-laden) DCs serve to enhance the probability of T cells encountering their cognate antigen[360,361]. These antigen-dependent interactions, known as 'clustering', are mediated by molecules such as intracellular adhesion molecules (ICAMs; also known as CD54) on DCs, binding to LFA-1 on naïve T cells, and DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), interacting with ICAM-2 and ICAM-3 on the T cells, and enable the T cells to efficiently probe/sample for the MHC- peptide complexes[362,363]. *In vitro*, T lymphocytes were shown to form (functional) synapses with DCs in the absence of both cognate antigen and MHC molecules, which promoted small $Ca2^{+}$ flux, limited proliferation and long-term survival of T cells, and T lymphocyte survival *in vivo* was dependent on the contact with MHC-II-expressing DCs[364,365]. Thus, even without recognition of their specific peptide during the initial interaction with DCs T cell receives a survival signal, and this mechanism potentially serves for long-term preservation of the diverse T cell/TCR repertoire required for efficient protective immune responses[352]. In addition, the recognition of the self-MHC peptide complex on DCs by TCR (in CD4-dependent manner) provides a tonic signal to T cell which does not induce cell activation but rather promotes its optimal responsiveness to the subsequent exogenous/foreign cognate antigen encounter[366].

Induction of T cell signalling relies on the integration of stimuli from several surface molecules and their subsequent conversion into complex intracellular signalling events, which eventually determine T cell response, including effective cell activation, anergy or apoptosis. On the immune response scale, these translate into the induction of response, either beneficial (pathogen elimination) and harmful (insufficient protective response/immunodeficiency or autoimmunity), or alternatively promote maintenance of immune tolerance/non-responsiveness.

According to the three-signal hypothesis, full activation of naïve $CD4^{+}$ T cells requires TCR engagement with its specific peptide presented in the context of MHC-II molecule by an APC (signal 1), the additional engagement of co-stimulatory molecules (signal 2) and stimulation with specific cytokines which determine the T lymphocyte differentiation towards a specific effector cell phenotype (signal 3)[367,368,369]. All three signals can be provided concurrently by a

mature DC. The exact mechanism by which TCR-mediated T cell activation occurs is unknown, but the current theories suggest several possibilities. Thus, the kinetic segregation model proposes that the spatial separation of membrane-spanning tyrosine phosphatase CD45 from the vicinity of TCR and its associated intracellular kinases would allow the accumulation of phosphorylated receptor residues and enable cell activation[370]. Kinetic proofreading model postulates that the different half-lives of the ligand binding to TCR enable the discrimination between self- and exogenous ligands, with the high-affinity (exogenous) ligands forming longer interactions with TCR to allow the number of biochemical modifications to occur to eventually trigger downstream signalling cascades[371]. Meanwhile, the conformational change model suggests that the MHC-II-peptide ligation to TCR promotes receptor conformation, which in turn triggers the exposure of the co-receptor CD3ε immuno-receptor tyrosine-based activation motifs (ITAMs), making those accessible for the tyrosine kinase phosphorylation and promoting the subsequent signalling events downstream[372]. Likely, the integration of these and multiple other theories is required to comprehensively explain the sensitivity, specificity, and dynamic nature of T cell activation.

The early membrane-proximal signaling events during T cell activation are initiated by leukocyte-specific tyrosine kinase (Lck), constitutively associated with TCR co-receptor CD4 intracellular domain[373,374]. Upon TCR engagement with MHC-II-peptide complex CD4 also comes in contact with MCH-II molecule, thus positioning Lck in proximity to the ITAM domains of the TCR-associated CD3 γ-,ε-,ζ- and δ chains and enabling their phosphorylation by the kinase[375]. Phosphorylated CD3 ITAMs recruit Syk family kinase Zeta-activated protein 70 kDa (Zap-70) which also undergoes Lck-mediated phosphorylation and activation, and in turn phosphorylates its target membrane-associated protein, linker for the activation of T cells (LAT)[375,376]. Activated LAT, in conjunction with other proteins, subsequently functions as a scaffold complex (signalosome) for the recruitment of multiple adaptor and effector signaling molecules, which trigger the key downstream signaling cascades, such as MAPK and NF-κB pathways[376]. The major consequences of the above signaling events include: actin and microtubule cytoskeleton reorganisation, required for the establishment of immunological synapses; integrin activation, which mediates stabilization of T

cell-APC contact; and rapid changes in expression of genes responsible for T cell polarization, proliferation and survival[377]. Several negative feedback loops are in place to fine-tune the signaling through TCR since the aberrant T cell activation can lead to the development of the autoimmune responses[377].

The initial engagement of TCR with its cognate peptide-MHC complex on the dendritic cell surface triggers an intricate sequence of T cell morphological changes and surface receptor re-organization, resulting in the formation of the stable molecular 'junction' at the interface between T cells and DCs termed immunological synapse (IS). Immediately upon T cell recognition of its cognate peptide-MHC complex, T cell halts its migration and forms transient microclusters at the primary contact site with DC, containing 30-300 TCR molecules along with kinases and adaptor proteins required for proximal singalling, as well as CD28 molecules and associated protein kinase C theta (PKCθ).[378,379] These TCR microclusters provide the initial signals for T cell activation, which are further sustained by a generation of new microclusters at the peripheral edges of the T cell, as it 'spreads' across the contact interface and engages more peptide-MHC complexes.[380,381] Following maximal 'spreading', T cell contracts and the centripetal flux of F-actin potentiates TCR microcluster to migrate towards the centre of the junction, dissociate from proximal signalling molecules, fuse into larger aggregates and eventually form central supramolecular activation cluster (cSMAC). In the mature IS, the cSMAC is divided into two distinct regions-$CD3^{hi}$ inner region containing a cluster of TCR molecules, and an outer $CD3^{lo}$ area composed of CD28 and PKCθ, along with negative regulators of T cell co-stimulation, CTLA-4.[382,383] While the inner cSMAC region is thought to represent a site of TCR signalling regulation through receptor internalisation and degradation, its outer edge enables to maintain persistent co-stimulatory/co-inhibitory signals essential for T cell activation fine-tuning.[379,382,384] The cSMAC is surrounded by a peripheral SMAC (pSMAC) domain enriched in integrin molecules LFA-1, which serves to enhance the T cell adhesion to DC and thereby increases its sensitivity to the antigen. [382,385] The outermost region of the immune synapse is known as distal SMAC (dSMAC), composed of molecules with large ectodomains, including CD45 tyrosine phosphatase, which is segregated from TCR to enable receptor activation.[385,386] The key function of the immune synapse is to maintain the stable cognate T cell-

DC contact for a prolonged period of time (5-12h), required for full naïve T cell priming.

In addition to efficient TCR engagement, additional co-stimulatory signals (signal 2) provided by accessory surface receptors are essential for complete T cell activation[387,388]. CD28 is a prototypical co-stimulatory surface receptor, constitutively expressed on the $CD4^+$ T lymphocytes but exhibiting low affinity to its ligands in the resting cell. This ensures that in the absence of antigen recognition and associated agonistic TCR stimulus the T cell is prevented from unnecessary activation[389]. Upon TCR stimulation, CD28 undergoes a conformational change and can bind its ligands CD80 (B7.1) and CD86 (B7.2) on the surface of mature DCs with enhanced affinity[390]. CD80 and CD86 expression is notably upregulated on antigen-bearing DCs during their maturation process[369,391]. Stimulated CD28 receptor, similarly to TCR, is phosphorylated on its cytoplasmic tail by membrane-proximal kinases, thereby recruiting a number of adaptor proteins and activating signaling pathways, which converge with TCR-induced intracellular cascades at the level of (aforementioned) signalosome/LAT-containing protein platform[392,393]. In this manner, the engagement of CD28 receptor results in the significant amplification of the signaling downstream of the TCR, and thus directly mediates the induction of naïve T cell proliferation, survival, and differentiation, the latter in conjunction with specific cytokine stimuli[394,395,395]. Such dominant co-stimulatory activity of CD28 requires tight regulation, mediated by cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) receptor proteins. CTLA-4 molecule, structurally related to CD28, is constitutively expressed on regulatory T cells but is upregulated on other T cells only following activation, and acts as a CD28 competitor for shared ligands CD80 and CD86, which it binds with 10-fold higher activity[396,397,398]. Mechanistically, CTLA-4 binding leads to TCR/CD28 signaling supression in the T cell (via recruitment of inhibitory phosphatases), as well as the transmission of immunosupressive signals to DCs through its ligands and the capture of the ligands from the DC surface by trans-endocytosis[399,400,401]. Biologically, CTLA-4 inhibition is an important checkpoint in regulation of T cell autoreactivity, exemplified by numerous reports associating mutations and polymorphisms in CTLA-4 with susceptibility to autoimmune disorders in humans[402,403,404]. Programmed death-1 (PD-1) receptor is another potent inhibitor

of T cell activation. Cytoplasmic tail of PD-1 associates with SHP2 phosphatase enzyme, which suppresses the activity of kinases mediating signaling downstream of TCR[405,406]. Thereby, PD-1 engagement on T cell can promote cell cycle arrest, anergy and/or apoptosis[407,408]. A range of other co-stimulatory proteins is involved in collectively orchestrating the subsequent T cell response, including ICOS, OX40, and CD40L on T cells and their respective ligands ICOS-L, OX40L and CD40 on DCs[409,410,411].

While the TCR signaling strength and co-stimulatory or co-inhibitory molecule engagement can influence T cell differentiation by favouring a commitment towards a certain lineage or impeding cell ability for differentiation, the critical role for T cell polarization towards distinct effector subsets is played by the APC/DC-derived cytokines (signal 3)[412,413,414,415]. The polarizing cytokine-induced signals are transduced (mainly) through the JAK/STAT pathway, and specific STAT proteins mediate the transcription of the lineage-determining /master transcription factors and lineage-associated genes. The development of the Th1 helper subset requires stimulation from both IL-12 and IFNy. While IL-12 is mainly produced by DCs, IFNy can be produced by differentiating Th1 cells themselves or by NK cells in response to DC-secreted IL-12[416]. The IFNy STAT1-mediated signaling induces the initial expression of T-box transcription factor (T-bet), a crucial master regulator of commitment towards Th1 lineage. In turn, T-bet enhances IFNy production by T cells, thus amplifying its own expression through the positive feedback loop, and induces IL-12Rß2 expression to sensitize cells to IL-12 stimulation[417]. In turn, IL-12-activated STAT4 also induces IFNy production to further promote sustained T-bet and IL-12Rß2 expression[418]. At the later stages of commitment, IL-18 production is induced through IL-12/STAT4 pathway, and IL-18 synergise with IL-12 for facilitating IFNy production by established Th1 cells[419]. The signals from both IL-4 and IL-2 are detrimental for naïve T cell commitment towards Th2 phenotype[419,420]. IL-4-induced STAT6 mediates the expression of GATA3, a master regulator of Th2 differentiation, and subsequently functions in concert with GATA3 to promote the lineage-specific gene expression[421,422]. IL-2 signaling, on the other hand, activates STAT5, which together with GATA3 coordinates (lineage-specific) IL-4 production and also promotes IL-4Rα expression[423,424]. Mature dendritic cells also provide IL-6 and TGFß, required for the early stages of T cell polarization into Th17

subset[425,426]. IL-6 and TGFß cooperate in inducing master transcription factor RORγt expression and subsequently IL-6-induced STAT3 cooperates with RORγt for induction of IL-17A (and IL-17F) production, as well as expression of IL-21 which further amplifies the above lineage-specific cytokine production[426]. Lastly, a combination of TGFß, secreted by mature DCs and IL-2, generated at early stages of T cell activation, is responsible for coordinating polarization into regulatory T cell (Treg) lineage[427,428].

1.3.2 Other outcomes of dendritic cell-CD4$^+$ T cell interaction

The requirement of at least two signals (Signal 1 and signal 2) for the naïve T cell activation and proliferation/clonal expansion are in place to ensure that the T cells respond only to the harmful exogenous antigens presented in the context of professional APCs, while remaining non-responsive to self-antigens. While the central tolerance mechanisms in the thymus govern the elimination of self-reactive T lymphocytes, the responsiveness to some antigens (e.g. developmental and food antigens) is not controlled by thymic selection thus allowing some autoreactive T cells to escape to the periphery. Therefore, a number of peripheral tolerance strategies are present to effectively suppress the priming/activation of such cells, including T-cell intrinsic mechanisms such as anergy, phenotypic skewing, and activation-induced cell death.[429]

1.3.2.1 Anergy

Anergy is a long-term state of cell hyporesponsiveness, which is induced following TCR recognition of the self-peptide in the absence of CD28 co-stimulatory signal.[430] Since the cooperative TCR/CD28 engagement is necessary for optimal recruitment of transcription factors essential for IL-2 expression, the anergic T cells exhibit notably reduced IL-2 production upon re-stimulation.[431,432]

Moreover, the anergic cells express anergy-associated molecules such as ubiquitin E3 ligases Itch and GRAIL, which facilitate degradation of the signaling components downstream of TCR, as well as specific transcription factors responsible for the maintenance of hyporesponsive cell state.[433,434]

The inhibitory signals delivered by CTLA-4 and PD-1 co-receptors were also implicated in promoting autoreactive T cell anergy. CTLA-4 was shown to be essential for anergy induction, as the naïve CTLA-4-deficient T cells exposed to tolerogenic stimulus *in vivo* and subsequently re-activated *in vitro* retained the ability for proliferation and IL-2 secretion, in contrast to wild-type T cells maintaining cell cycle arrest[435]. Moreover, mice deficient in CTLA-4 develop a fatal autoimmune multiorgan disorder, further highlighting the crucial negative regulatory role of CTLA-4[436]. Another critical co-receptor, PD-1, functions by promoting dephosphorylation of membrane-proximal TCR-induced signaling molecules and interfering with downstream signaling cascades. Thus, the ligation of PD-1 on the autoreactive T cell leads to diminished cell proliferation and increased susceptibility for apoptosis, thereby ultimately inhibiting the expansion of self-reactive naïve T cell and their potential for acquiring an effector phenotype[437]. In line with that, the blockade of PD-1 engagement with one of its receptors, B7-H1, resulted in the reversal of T cell anergy, while the disruption of PD-1 gene in mice manifested in spontaneous lupus-like autoimmune phenotype development[438,439].

While being crucial in mediating T cell activation to the harmful antigens, DCs are also suggested to promote induction of non-responsive state in T cells. Such 'tolerogenic' DCs, presenting self-antigens, usually exhibit an immature or semi-mature phenotype with characteristic low-level expression of both MHC-peptide complexes and co-stimulatory molecules insufficient for optimal T cell activation[440]. In addition, some mature DC subsets were also described to promote T cell anergy as well as regulatory T cell generation. The suggested mechanisms of 'tolerogenic' DCs involve the delivery of inhibitory signals through expression of PD-1 receptors PD-L1 and PD-L2, secretion of anti-inflammatory cytokines IL-10 and TGF-β, which potentiate generation of regulatory T cells, as well as Indoleamine 2,3-dioxygenase (IDO) secretion,

leading to T cell nutrient deprivation and also limiting T cell clonal expansion.[441,442,443]

1.3.2.2 Activation-induced cell death (AICD)

Along with anergy, activation-induced cell death (AICD) represents another fundamental mechanism for maintaining peripheral self-tolerance. AICD specifically occurs in $CD4^+$ T cells repeatedly stimulated through their TCR *in vitro*, which are thought to represent the autoreactive chronically activated cells (escaping thymic selection) and repeatedly encountering their cognate self-peptides in the periphery.[444] The repetitive antigenic stimulation induces $CD4^+$ T cells to upregulate the expression of the death receptor Fas (CD95) and its ligand, with the subsequent Fas-FasL interaction mediating the formation of death-inducing signalling complex (DISC) and induction of caspase cascade, which ultimately leads to cell death by apoptosis.[445,446,447] AICD can occur either through Fas-FasL interaction between the two cells (fratricide) or as a cell 'suicide', whereby the soluble FasL binds its receptor in an autocrine manner.[448,449] The primary evidence of the connection between Fas-mediated AICD and immune tolerance came from *lpr* and *gld* mice bearing deactivating mutations in Fas and FasL genes, respectively, exhibiting failure in thymocyte selection, which manifested in lymphadenopathy and systemic lupus-like autoimmunity.[450] However, the subsequent study demonstrated that Fas deficiency did not affect negative thymic selection but instead rendered mature peripheral T cells resistant to apoptosis, thus highlighting the importance of Fas-mediated AICD in specifically maintaining T cell peripheral tolerance.[451]

1.3.3 Evidence of the DC-CD4$^+$ T cell crosstalk importance in RA

Given the crucial role of DCs in the induction and fine-tuning of T cell responses, the alterations in their activation status, quality of antigen presentation and co-stimulatory signal transmission can modulate the outcome of the DC-CD4$^+$ T cell interaction and contribute to the development of multiple autoimmune conditions, including RA. The strongest evidence is provided by the increased RA susceptibility in individuals bearing one or several alleles of the HLA-DRB1 gene (including HLA-DRB1*0401, *0404 and *0405), which harbour a 5 amino acid motif ('shared epitope') within a peptide-binding groove pocket of the HLA-DRB1 MHC-II molecule. The 'shared epitope'-containing HLA-DRB1 molecules exhibit an increased propensity towards binding and presentation of citrullinated self-peptides, and the recognition of such peptides by CD4$^+$ T cells, reported in both mice and RA patients, leads to pro-inflammatory cytokine production by T cells and might also be linked to T cell-dependent B cell activation for ACPA antibody generation.[452,453] Thus, the preferential presentation of modified self-epitopes by DCs (and other APCs) appears to have an important role in disease pathogenesis. Other RA-associated allelic polymorphisms in genes including *REL*, IRAK1, NFKBIE and CCL21 can potentially affect DC maturation and migration to the lymph nodes, and thereby further contribute to alteration of their T-cell stimulatory capacity.

A more direct piece of evidence on the relevance of DC-CD4$^+$ T cell interaction in the disease, in particular during the onset of autoimmune response, was obtained from the murine models of experimental RA. Thus, Brewer *et al.* demonstrated that the transfer of mature DCs, presenting collagen-derived peptide, into the footpads of recipient DBA/1 mice was sufficient for initiation of erosive RA in the adjacent joint, with the induction phase corresponding with the priming of endogenous collagen-specific CD4$^+$ T cells and their differentiation into Th1 subset.[454] In a model of acute early RA, developed in our lab, the transfer and repeated cognate re-activation *in vivo* of CD4$^+$ Th1 cells of joint-irrelevant specificity created an inflammatory joint environment, which was proposed to promote the presentation of self-antigen (collagen II) to the

endogenous autoreactive $CD4^+$ T cells by DCs in immunogenic fashion, leading to the self-tolerance breakdown.[455] Indeed, a follow-up study demonstrated the endogenous conventional DC subset being activated and presenting antigen prior to the development of anti-collagen $CD4^+$ T cell responses. In line with that, the selective depletion of conventional DCs in CD11cDTR mice by *in vivo* administration of diphtheria toxin prior to mice receiving secondary antigenic challenge lead to abrogation of autoreactive (collagen-II-specific) T cell responses and associated breach of self-tolerance, and resulted in amelioration of joint pathology characteristic for the model.[456]

On the other side of the interaction, T cells in RA exhibit altered threshold of the TCR signaling and associated/consequent aberrations in cell activation. For instance, increased phosphorylation of extracellular-signal-regulated protein kinase (ERK), a member of the pivotal signaling pathway downstream of TCR, is observed in RA patient T cells, which appears to promote sustained TCR-mediated signaling in response to suboptimal antigenic stimulation[457]. Genetic polymorphisms associated with RA were also identified at loci of CD28, CTLA-4, protein tyrosine phosphatase, non-receptor type 22 (PTPN22) and CD247 (TCRζ), which encode molecules mediating membrane-proximal TCR signalling events and is thus involved in regulating TCR stimulation threshold. Polymorphism(s) in PTPN22 gene product Lyp phosphatase, particularly strongly linked to RA susceptibility, results in Lyp failure to bind its partner kinase Csk and thereby abrogates Lyp capacity to negatively regulate the activity of proximal TCR signaling intermediates[458]. Thus, the perturbations in dendritic cell capacity for T cell stimulation in combination with altered T cell responsiveness in RA-predisposed individuals result in aberrant cell crosstalk, which can occur during naïve $CD4^+$ T cell priming in periphery but can also take place during the self-reactive thymocyte elimination in the thymus. Given all the above, DC-$CD4^+$ T cell interactions are considered indispensable in both breakdown of tolerance and initiation of adaptive immune responses in RA and represent an important ‘immune checkpoint’ to be targeted therapeutically.

Thus, additional evidence is provided by some successful therapeutic approaches designed to interfere with or modulate DC-$CD4^+$ T cell crosstalk. One of such therapeutic modalities is CTLA-4 molecule homologue abatacept, comprised of

extracellular human CTLA-4 domain and modified Fc portion of human IgG1. Similarly to an endogenous CTLA-4, abatacept competes with CD28 for binding to CD80/86, thereby delivering a co-inhibitory signal and effectively suppressing T cell activation by APCs/ dendritic cells.[459] In patients with different stages of RA and treatment background abatacept is reported to effectively reduce disease activity, improve physical function and halt the radiographic damage progression.[212,211,213] The strategy of other therapeutic approach, tolerogenic DC (tolDC) therapy, involves in vitro generation of semi-mature 'tolerogenic' DCs which have the potential to induce hyporesponsive/anergic state in autoreactive T cells. In the initial clinical study in early RA patients, a single intradermal injection of tolDCs exposed to citrullinated peptides was administered, which lead to a reduction in effector T cells, increase in the regulatory-to-effector T cell ratio and diminished T cell responsiveness to citrullinated peptides, along with improvement in disease activity scores[460]. Another clinical study, AutoDECRA, used synovial fluid as the source of self-antigens for presentation by tolDCs and used intra-articular administration for cell delivery. The tolDC administration improved the local disease activity in a proportion of patients but did not induce any systemic immunomodulatory effects[461]. Thus, it appears that DCs with tolerogenic phenotype can modulate autoreactive effector T cell responses by inducing anergy and/or potentially promoting apoptosis in RA patients[461]. These observations further support the importance of DC-$CD4^+$ T cell interactions in RA, as the therapeutic blockade or modulation of this crosstalk leads to diminished autoreactive T cell responses and subsequent clinical symptom improvement.

1.4 Dynamics of DC-$CD4^+$ T cell interactions during RA development

The process of RA pathogenesis can be described to occur in several phases: genetically and environmentally-determined 'at risk' phase, preceding the initial loss of self-tolerance; initiation and propagation of autoimmune response, followed by immune cell infiltration into the joint; and transition to chronic

inflammation, which eventually culminates in joint pathology and symptomatic disease. Extensive research utilizing murine models was conducted to determine the contribution of specific cells and cell-cell cross-talk into each of these disease stages, with the aim to gain better insights into immune mechanisms underlying the RA progression and accordingly advance the therapeutic disease management. A number of studies specifically investigated the potential contribution of initial DC-$CD4^+$ T cell interaction (i.e. cognate $CD4^+$ T cell priming) to the inciting phase of the pathology. Thus, in a mouse model of acute early RA (described in detail in ref.[455] and Chapter 5), the self-tolerance breakdown event and associated autoreactive $CD4^+$ T cell response development were directly preceded by conventional DC activation and antigen presentation in the joint-draining lymph nodes, suggesting these cells to be responsible for priming of the self-reactive $CD4^+$ T lymphocytes.[456] The depletion of these conventional DCs by administering diphtheria toxin to CD11c-diphtheria toxin-receptor (DTR) mice prior to administration of antigen triggering self-tolerance breakdown appeared to prevent self-reactive $CD4^+$ T cell response generation, further confirming the indispensable requirement of cognate naive $CD4^+$ T cell-DC engagement for triggering the autoimmune response in this model.[456] Andrew Cope and colleagues took a different approach and assessed the consequences of $PTPN22^{R620W}$ mutation, a single nucleotide polymorphism most strongly associated with genetic susceptibility to RA, on the quality of DC-mediated T cell responses. They elegantly demonstrated mice harbouring orthologous $PTPN22^{R619W}$ mutation to exhibit an enhanced expansion of the conventional DC2 population, which was sufficient to augment T cell proliferation and T follicular helper cell generation. The authors thereby proposed that perturbations in DC homeostasis, driven by PTPN22 polymorphism, might be causal of aberrant autoreactive T cell priming and initiation of autoimmunity in RA. [462] In another study, the absence of PTPN22 in DCs resulted in enhanced immune complex-derived antigen presentation and $CD4^+$ T cell-DC conjugate formation, which subsequently augmented $CD4^+$ T cell proliferation. These results provide an additional explanation on how $PTPN22^{R620W}$ mutation in a specific context might contribute to dysregulated DC-$CD4^+$ T cell interactions and potentiate autoimmunity initiation.[463] Apart from the initial triggering of autoimmunity, the continuous (new) naïve $CD4^+$ T cell activation by DCs is thought to crucially contribute to the propagation of the autoimmune response in RA through the

mechanism of epitope spreading. Epitope spreading is a process of diversification of T cell as well as B cell responses from a dominant antigenic epitope, triggering the initiation of the autoimmune response, to the recognition of other epitopes of the dominant antigen (generated by post-translational modification in the inflamed milieu) as well as distinct antigenic molecules, likely exposed following inflammation-associated tissue damage. In contrast to other autoimmune diseases (e.g. multiple sclerosis), the animal-model based research into mechanisms underlying epitope spreading phenomena and its contribution to the pathology development in RA is fairly limited. Khmaladze and colleagues employed a knock-in B10Q.ACB mouse strain, which spontaneously developed high-frequency autoreactive B cells specific for C1 epitope of collagen II (CII) but remained protected from collagen-induced arthritis (CII), and introduced a mutation (to *Ncf1* gene) leading to deficiency in reactive oxygen species (ROS). This ROS deficiency appeared to break the resistance to CIA, and the disease progression and severity correlated with the development of antibodies specific for additional, non-C1 CII epitopes. The Ncf1-deficient mice also exhibited enhanced CII-specific T cell responses, suggesting that autoreactive B cells might have potentially received T cell help for enhanced response generation and possibly epitope spreading.[464] Interestingly, in a hGPIc peptide-induced (GIA) murine arthritis model which was subjected to regulatory T cell depletion, Yang and colleagues observed not only B cell but also pronounced T cell response diversification, evidenced by detection of antigen-specific T cells recognising several collagen type II epitopes, unrelated to disease-inciting peptide, which possibly contributed to the disease perpetuation.[465] The likely mechanism of T cell epitope spreading requires initial $CD4^+$ T cell priming by an APC (DC) presenting immunodominant epitope, with subsequent migration of activated $CD4^+$ T lymphocyte to the target tissue, where it might be reactivated by the same (primary) epitope and release cytokines and chemokines. These will promote recruitment and activation of innate immune cells (e.g. macrophages), which in turn promote tissue damage through release of proteolytic enzymes and ROS. The neoepitopes generated from the tissue debris can then be presented by APCs (DCs) to 'new' naïve autoreactive $CD4^+$ T cells in the lymph node, leading to further recruitment of additional activated CD4+ T cells and tissue destruction, thereby creating a self-perpetuating cycle [466] Thus, the continuous autoreactive $CD4^+$ T cell priming by DCs, underlying epitope spreading, plays an

important role in disease perpetuation and likely contributes to the chronicity of autoimmune response in RA. Along with the newly primed $CD4^+$ T cells, the T cells of activated phenotype detected in the synovium of RA patients might also contribute to the progression and chronicity of autoimmune response. However, whether these cells specifically require interaction with DCs for secondary activation is largely unknown, as they could also be re-activated in an antigen-specific manner by other APCs abundantly present in the joint, as well as undergo cytokine-dependent bystander activation which does not require TCR engagement. One of the clues suggesting the DC involvement in antigen presentation in the RA joint is the enrichment of the synovium compared to peripheral blood/circulation, with myeloid subset DCs of a mature phenotype expressing MHC-II and co-stimulatory molecules, which were capable of effective autologous T cell stimulation.[467] Moreover, the presence in the synovial fluid of myeloid progenitors along with soluble factors capable of promoting their differentiation suggested that mature DCs can be generated locally within the joint and can also participate in the amplification of inflammation by joint-infiltrating autoreactive $CD4^+$ T cell (re-)stimulation. The direct evidence of the activated antigen-specific $CD4^+$ T cell re-activation in the joint comes from an elegant study by Benson *et al*. which employed the aforementioned 'breach-of-tolerance' RA mouse model. The study demonstrated the adoptively transferred OT-II $CD4^+$ T cells of activated phenotype to infiltrate the joints following the secondary challenge with their cognate antigen (HAO) *in vivo*, and, using intravital microscopy, observed these cells within the articular environment to form prolonged interactions with endogenous $CD11c^+$ DCs, consistent with the antigen-specific recognition and activation.[468] Overall, while the evidence is very limited, these individual cues allow proposing that the antigen-experienced $CD4^+$ T cells infiltrating the RA synovium might at least partially rely on cognate interactions with DCs to receive re-activation signals. Thus, the cognate $CD4^+$ T cell-DC interactions likely occur throughout the course of RA development, thereby highlighting their importance in the disease pathogenesis as well as emphasizing the value of this cell interaction for therapeutic modulation.

1.5 Hypothesis and aims

A number of studies demonstrate the effect of tofacitinib on $CD4^+$ T cell ability to proliferate and produce pro-inflammatory cytokines. Employing the antigen-specific cell system, I wanted to specifically investigate the impact of the drug on $CD4^+$ T cell interaction with its cognate antigen-presenting DC. I also aimed to further dissect whether the drug exerts the effect during the naïve $CD4^+$ T cell priming or interferes with antigen-experienced $CD4^+$ T lymphocyte re-activation, both of which play a crucial part in initiation and propagation of RA pathogenesis. Based on the ability of tofacitinib to inhibit cytokine signalling through the JAK/STAT pathway, I hypothesised that the drug would impact the efficiency of $CD4^+$ T cell-DC primary interaction and lead to the alterations in $CD4^+$ T cell phenotype and function. With the efficacy of tofacitinib on RA pathology being so far studied and confirmed in the murine models of established RA, I intended to determine if similar improvements in the disease pathogenesis could be achieved by introducing the drug very early in the disease, in line with the current trends for therapeutic interference at the onset of RA.

Thus, the key aims of this project were:

- To determine the effect of tofacitinib treatment on $CD4^+$ T cell-DC interaction efficiency and functional outcome *in vitro* and *in vivo*

- To determine at what stage of the $CD4^+$ T cell-DC interaction, priming or re-activation, does tofacitinib have an impact

- To assess the drug impact on disease pathogenesis in the ‘breach of tolerance’ early acute RA mouse model

2 Materials and Methods

2.1 Buffers and reagents

2.1.1 Complete medium

RPMI-1640 (Life Technologies) supplemented with 10% foetal bovine serum (FBS) (Gibco), L-glutamine (Gibco) and Penicillin/Streptomycin (Sigma-Aldrich).

2.1.2 FACS buffer

Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies) supplemented with 2% foetal bovine serum,1 mM EDTA, and 0.01% Sodium azide.

2.1.3 MACS buffer

Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies) with 1% foetal bovine serum and 2 mM EDTA.

2.1.4 JAK inhibitor tofacitinib

Tofacitinib free base and tofacitinib citrate salt were purchased from LC Labs, reconstituted in dimethyl sulfoxide (DMSO)(Sigma), and stored as single-use aliquots at -20 C. DMSO was serially diluted to achieve the concentration of 0.1% or 0.001% in the cell culture, corresponding to the two tofacitinib concentrations used.

For administration to mice by oral gavage, tofacitinib citrate stock was further dissolved in sterile-filtered DPBS containing 0.5% Methylcellulose (Sigma) and 0.025% Tween20 (Sigma) at the concentration of 6.25 mg/ml and sonicated for 5 minutes. DMSO was utilized as a vehicle control at a maximal non-toxic dilution of 5 % dissolved in the same solution as the drug.

2.2 Mice

2.2.1 **C57BL/6**

Male C57BL/6 mice were purchased from Harlan laboratories UK (currently ENVIGO) at 6 weeks of age and acclimatised for 1 week prior to their use for any procedures. C57BL/6 mice were used between 6-12 weeks of age as donors of bone marrow or adoptive transfer recipients.

2.2.2 **CD45.1 OT-II**

OT-II T cell receptor (TCR) transgenic mice were bred in-house (Central Research Facilities, University of Glasgow, UK). The majority of the T cells of OT-II mice express αßTCR specific for chicken ovalbumin peptide (OVA) $^{323-339}$ presented in the context of MHC-II molecule[469]. *In vitro* co-culture of OT-II T cells with OVA$^{323-339}$ -pulsed dendritic cells generates robust T cells response and allows to monitor changes in this antigen-specific interaction. Our OT-II mice also express CD45.1 on all leukocytes, which is a pan-leukocyte marker with two allelic variants-CD45.1 and CD45.2-bearing the same function. This system allows discriminating the cells transferred from the CD45.1 OT-II donor into CD45.2 C56BL/6 recipient by flow cytometry and manipulating them by introducing OVA$^{323-339}$ peptide into the recipient. T cells from OT-II mice were used for *in vitro* co-cultures and adoptive transfer experiments.

All animals were maintained on a 12/12-hour light/dark cycle with water and food ad libitum, and all the procedures were performed in accordance with local ethical and UK Home Office regulations.

2.3 Isolation and preparation of cells

2.3.1 CD4$^+$ T cell isolation from mouse lymph nodes and spleen (StemCell EasySep kit)

Lymph nodes and spleens were harvested from OT-II transgenic mice, passed separately through a 70 µm EASYstrainer (Greiner Bio) and cell suspension collected into complete media. Cells were spun at 400 g for 5 min at 4^{o}C. Cell pellets from the spleens were then treated with 1xRBC lysis buffer (eBioscience) for 5 minutes at room temperature (RT), washed with complete media, and pellets from lymph nodes and spleen were pulled together for counting. Cell viability and number were determined using Trypan blue (Sigma) exclusion method and a haemocytometer. Cells were then resuspended in isolation buffer (DPBS with 2% foetal bovine serum and 1 mM EDTA) at 1x10^8 cells/ml and CD4$^+$ T cells were isolated using EasySep Mouse CD4$^+$ T cell isolation kit (Stemcell Technologies) following manufacturers' instructions. In brief, 50 ul of rat serum per 10^8 total cells was added along with 50 ul per 10^8 total cells of Isolation cocktail, containing biotinylated antibodies labelling CD4$^-$ cell fraction. Cells were incubated for 10 minutes at RT and 75 ul per 10^8 total cells of streptavidin-coated magnetic spheres were then added for another 2.5 minutes. The CD4$^-$ cells labelled with antibodies and magnetic spheres were then removed from the cell suspension by incubation in the EasySep magnet for 2.5 minutes, and the purified CD4$^+$ T cell fraction was collected into a fresh tube for counting and CFSE labelling.

Cell viability and number were determined as previously described, and the cells resuspended at 10^7 cells/ml in RPMI-1640 (Life Technologies) with 2% FBS for CFSE staining. 5 mM CFSE stock dissolved in DMSO (both- Life Technologies) was diluted to 1 mM in DPBS and then added to cell suspension at 1 uM concentration. Cells were stained for 5 min at 37C in the dark, washed twice with complete media, and assessed for viability and number as before. Cells were then resuspended at 2.5x10^6 cells/ml in complete media for co-culture assays.

2.3.2 **$CD4^+$ T cells isolation (MACS kit) and Th1 polarization**

Lymph nodes and spleens were harvested from 5 OT-II transgenic mice, along with spleens from 4 C57BL/6 mice, and were passed separately through a 70 µm EASYstrainer (Greiner Bio). The strainers were washed with MACS buffer and the collected cells spun down at 400 g for 5 min at 4°C. Cell pellets from the spleens were individually treated with 1xRBC lysis buffer (eBioscience) for 5 minutes at room temperature (RT), washed with complete media, and the cells from OT-II mouse spleens and lymph nodes were then resuspended in MACS buffer and pulled together. Splenocytes from C57BL/6 mice were resuspended in MACS buffer and kept on ice. Cell viability and number of cells from OT-II mice were determined as described above, and the cells were resuspended in MACS buffer at 10^7 total cells per 40 ul for $CD4^+$ T cell isolation using MACS $CD4^+$ T cell isolation kit (Miltenyi Biotech). In brief, 10 ul of the biotin-antibody cocktail was added per 10^7 total cells, mixed and incubated in the fridge for 5 min. MACS buffer was then added at 30 ul per 10^7 total cells, followed by anti-biotin magnetic Microbeads at 20 ul per 10^7 total cells. While the cells were incubated for 10 min in the fridge, LS column (Miltenyi Biotec) was set up in the magnetic field of a MACS Separator (Miltenyi Biotec) and washed with 3 ml of MACS buffer. The cell suspension was then filtered through 70 µm EASYstrainer (Greiner Bio) and placed in LS column (Miltenyi Biotec), which retained magnetically labelled $CD4^-$ cells and allowed OT-II $CD4^+$ fraction to flow through to be collected. The column was washed with 3 ml of MACS buffer to collect the remaining $CD4^+$ T cells, then removed from the separator and flushed out with MACS buffer, using a plunger, to collect the magnetically labelled APCs into the sample containing C57BL/6 splenocytes. Both OT-II cells and C57BL/6 splenocytes were resuspended in complete media and counted. Splenocytes were then treated with 500 ul of mitomycin C (1 mg/ml stock in DPBS; Sigma) at 37°C for 45 min to inhibit their ability to proliferate. Thereafter, splenocytes were washed twice in complete media and combined with OT-II $CD4^+$ T cell at 1:4 ratio in 200 ml of complete media supplemented with ovalbumin chicken peptide ($OVA^{323-339}$)(1 ug/ml), IL-12(R&D Systems) (10 ng/ml) and anti-IL-4 antibody (BioXCell)(2 ug/ml). The resulting cell suspension was split into 75cm^2 tissue culture flasks (Corning) at 50 ml/flask and incubated at 37°C 5% CO_2 for 72 h.

After 72h, the media in the flasks changed its colour to the peachy pink and the large clumps of proliferating cells were observed. Cells from all flasks were pulled together, blasting cells counted and 3 ml of suspension was used for FACS staining to determine the percentage of the OT-II Th1 polarized cells obtained. The cells were stained according to the FACS surface staining protocol (see section 2.5.1) for viability, CD4, Vα2 and Vβ5 markers (Table 2-1) and the percentage of $V\alpha2^+V\beta5^+$ double-positive cells (from the $CD4^+$ population) was used to calculate the total number of OT-II Th1-polarized cells obtained. The cells were resuspended at 10 x 10^6 cells/ml in sterile DPBS and utilized for the breach-of-tolerance RA mouse model induction.

2.3.3 **Dendritic cell generation from the mouse bone marrow**

Femur and tibia bones from both mouse legs were harvested and cleaned from the surrounding tissue, the epiphysis cut off from both sides of each bone and the bone marrow flushed out with complete media using a 25G syringe. The marrow was then passed through a 70 μm EASYstrainer, a strainer washed with complete media, and a single cell suspension collected and spun at 400 g for 5 min at 4ºC. Red blood cells were removed by incubation with 1xRBC lysis buffer (eBioscience) for 5 minutes at room temperature (RT), cells washed with complete media and counted. Cells were then resuspended at 1.5 x 10^6 cells/ml in complete medium containing 5% GM-CFS supernatant and seeded at 1.5 x 10^6 cells/well in 6-well culture plates (Corning) with additional 2 ml of 5% GM-CSF in complete medium. Cells were cultured at 37ºC 5% CO_2. On day 3, each well was supplemented with 2 ml of 5% GM-CSF in fresh complete medium. On day 6 of culture, medium from all wells was aspirated and 3 ml of fresh complete medium containing 5% GM-CSF were added. Cells also received 100 ng/ml of bacterial lipopolysaccharide (LPS) from Escherichia *coli* (E.*coli*) strain 0111:B4 (Sigma), with control wells left unstimulated, and cultured overnight. On day 7, $CD11c^+$ dendritic cells from an LPS-stimulated and control wells were assessed by FACS for the expression levels of MHC-II and co-stimulatory molecules CD40 and CD86 as indicators of their mature phenotype. Cultures containing ≥80% of

$CD11c^+$ cells which upregulated the above molecules upon LPS stimulation were considered of satisfactory quality and used for *in vitro* assays.

2.4 *In vitro* cultures

2.4.1 BMDC-$CD4^+$ T cell co-culture

On day 6 of culture, BMDCs were treated with 100 ng/ml of LPS (from E.coli 0111:B4, Sigma) overnight to promote their maturation, followed by incubation in the presence of either 0.1 ug/ml or 5 ug/ml of $OVA^{323-339}$ peptide (Sigma) for 2 h at 37°C 5% CO_2. $CD4^+$ T cells were isolated from lymph nodes and spleens of OT-II mice using EasySep kit (Stemcell Technologies) and labelled with CFSE to monitor their proliferation. BMDCs ($0.2x10^5$) were co-cultured with $CD4^+$ T cells ($2x10^5$) in round-bottom 96-well plates (Corning) for 24 or 72 h in the presence of either 100 nM or 10 000 nM of Tofacitinib (base or citrate forms) (LC Labs) or corresponding concentrations of DMSO (Sigma).

2.4.2 BMDC-$CD4^+$ T cell re-challenge co-culture

BMDCs were treated as described above and the $CD4^+$ T cells were isolated from lymph nodes and spleens of OT-II mice using EasySep kit (Stemcell Technologies). BMDCs ($0.2x10^5$) were co-cultured with $CD4^+$ T cells ($2x10^5$) in round-bottom 96-well plates (Corning) for 72 h in the presence of either 100 nM or 10 000 nM of Tofacitinib base (LC Labs) or corresponding concentrations of DMSO (Sigma). The cells were then washed twice in complete media, $CD4^+$ T cells counted and rested in complete media supplemented with 1 ng/ml of IL-2 (Biolegend) for another 72 h. $CD4^+$ T cells were subsequently CSFE-labelled and each treatment group split in half and co-cultured with fresh BMDCs in the presence or absence of OVA peptide for the final 72 h.

On other occasions, OVA-pulsed BMDCs ($0.2x10^5$) were co-cultured with $CD4^+$ T cells ($2x10^5$) for 72 h, $CD4^+$ T cells rested in the presence of 1 ng/ml of IL-2 (Biolegend) for 72 h and CFSE-labelled. The final step involved incubation with fresh OVA-treated BMDCs in the presence of an appropriate concentration of Tofacitinib base (LC Labs) or DMSO (Sigma).

2.4.3 **αCD3/CD28 $CD4^+$ T cell activation assay**

96 well round-bottom plates (Corning) were coated with anti-CD3 antibody diluted in DPBS at 1 ug/ml and incubated overnight at 4°C. Control wells received the corresponding volume of DPBS alone. The next day, $CD4^+$ T cells were isolated from lymph nodes and spleens of OT-II mice using EasySep kit (Stemcell Technologies) as described previously, viable cells counted and resuspended in complete media. Plate with anti-CD3 antibody solution was blotted dry and 150 ul ($3x10^5$) of $CD4^+$ T cell suspension were added to both control and antibody-coated wells. Antibody-coated and control wells also received 50 ul of anti-CD28 (NA/LE) (BD Biosciences) diluted in complete media or complete media alone, respectively, to obtain a final concentration of 5 ug/ml in the well. Cells received increasing concentrations of tofacitinib base (LC Labs) in DMSO or corresponding DMSO concentrations to set up a dose-response assay and were incubated for 48 h at 37 °C.

2.5 Western blotting

2.5.1 BMDC monoculture and cytokine stimulation

Dendritic cells were generated as described above, except for the LPS stimulation. Instead, on day 7 cells were harvested, counted, resuspended at $1x10^6$ cells/ml in complete media, and seeded at $1x10^6$ cells/well in a 12-well culture plate (Corning). Cells were treated with either 0.001% of DMSO (Sigma) or 100 nM of Tofacitinib base (LC Labs), with 3 wells per condition, and 6 wells were left untreated. After overnight incubation at 37°C 5% CO_2, half of the untreated cells together with cells receiving DMSO or Tofacitinib were stimulated with a 100 ul of cytokine cocktail (see below) for 15 mins at 37°C. Immediately after the cells were processed for analysis by Western blotting.

Stimulant	Final dilution (in the well)	Source
PMA	10 ng/ml	Sigma
Ionomycin	500 ng/ml	Sigma
IL-4	20 ng/ml	R&D Systems
IL-6	20 ng/ml	Biolegend
IFNy	25 ng/ml	Peprotech
GM-CFS	200 ng/ml	R&D Systems

2.5.2 **BMDC - CD4$^+$ T cell co-culture**

Dendritic cells were stimulated with LPS on day 6 of culture and half of them also pulsed with OVA$^{323-339}$ peptide on day 7 for 2 hours, as described above (section 2.4.1). Cells stimulated with LPS only or both LPS with OVA peptide were collected separately, spun at 400 g for 5 min, and resuspended at 0.2x10^6 cells/ml in complete media. CD4$^+$T cells were purified using the EasySep kit (Stemcell Technologies) and resuspended at 2x10^6 cells/ml in complete media. The co-culture was set up with 0.1x10^6 dendritic cells and 1x10^6 CD4$^+$T cells per well in a 24-well co-culture plate. T cells were co-cultured with DCs in the presence or absence of OVA peptide, and both groups received either 0.001% of DMSO (Sigma) or 100 nM of Tofacitinib base (LC Labs) treatment overnight. The positive control wells contained OVA pulsed dendritic cells and CD4$^+$T cells and received 20 ng/ml of IL-6 (Biolegend). Cells were rested overnight at 37^{o}C 5% CO_2 and processed for analysis by Western blotting.

2.5.3 **Sample preparation**

After overnight incubation, dendritic cells were washed in ice-cold PBS and lysed on ice in 40 ul of ice-cold RIPA buffer (Sigma) containing Halt protease inhibitor cocktail (Thermo Fischer). For co-culture samples, supernatants containing T cells were collected and dendritic cells were scraped from the wells and added to the supernatants. Supernatants were centrifuged at 12,000 rpm for 15 min and cell pellets lysed in RIPA buffer (Sigma) containing Halt protease inhibitor cocktail (Thermo Fischer). Resulting cell lysates were lysed for further 30 mins in the cold room (at 4^{o}C) with rotation. Cells were then centrifuged at 12,000 rpm for 15 min (at 4^{o}C) and supernatants collected into fresh tubes. Pierce BCA protein assay kit (Thermo Fischer) was used to determine protein concentration for each sample and the loading volumes were adjusted to have equal amounts of total protein across samples. Lysates were mixed with 5xSDS loading buffer at

4:1 dilution, boiled at 95ºC for 5 mins and then kept on ice for immediate blotting or stored at -20ºC for future use.

2.5.4 **Immunoblotting and protein detection**

RunBlue SDS 4-20% pre-cast gel (Expedeon) was set up in a buffer tank filled with running buffer (Expedeon). Equal amounts of protein from each sample were loaded onto the gel along with 7 ul of PageRuler Pre-stained protein ladder (Thermo Fischer). Proteins were separated by PAGE at 180 V for 45 mins and transferred onto 0.45 um nitrocellulose membrane (Amersham) in a transfer buffer (Biorad) at 100 V for 1 hour. The membrane was then washed on a shaker platform in 1xTBS buffer for 5 mins at RT and incubated with blocking buffer (5% dry milk in 1xTBS-T) for 1 h at RT to avoid non-specific antibody binding. The membrane was subsequently washed twice for 5 mins with 1xTBS-T with agitation and blotted overnight at 4ºC with rotation in dilution buffer with primary antibodies recognising STAT3 or pSTAT3 (Cell Signalling Technology). The washing step was repeated, followed by incubation for 1 h at RT with species-specific detection antibodies (for antibodies-see Table 2-2) in 5% dry milk in 1xTBS-T. After the final wash, specific proteins were detected with WesternBright ECL HRP Substrate reagent (Advansta) according to manufacturers' instructions and visualized using C-DiGit scanner and Image Studio Lite software (Licor Biosciences). After detection, the membrane was washed as previously, and the antibody incubation and detection steps repeated to assess β-actin protein levels.

2.6 Flow cytometric cell analysis

2.6.1 FACS staining for T cell surface markers

Following 24 or 72 h incubation in co-culture with DCs, cells were washed in DPBS and stained for viability using fixable viability dye (Thermo Fischer) for 20 mins at RT, followed by incubation with FC-receptor block (2.4G hybridoma supernatant) for 10 min at 4 °C. The activation status of T cells was subsequently assessed using antibodies against CD4, CD69, CD44, and CD62L (see Table 2-1 for a list of flow cytometry antibodies). Samples were washed with FACS buffer between each incubation step at 400 g for 3 mins, except for after FC blocking step, and the supernatants were discarded. After the final wash, samples were transferred through 40 µm nitex mesh into polystyrene FACS tubes and acquired using a BD LSR FORTESSA or BD LSR II Analyser (both Becton, Dickinson, and company). Data analysis performed using FlowJo software (Tree Star Inc).

2.6.2 $CD4^+$ T cell PMA/Ionomycin stimulation and intracellular cytokine staining

Cells from 72 h co-culture were washed and stimulated directly in the culture plate. For *ex vivo* stimulation of popliteal LNs, tissues were mashed through a nitex into a cell suspension and samples spun down in a 96-well plate. Stimulation cocktail was made up in complete media with PMA (10 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma) and GolgiStop protein transport inhibitor (1:1000 dilution; BD Biosciences) and samples stimulated for 4 h at 37 °C. Cells were subsequently washed in DPBS, stained for viability and cell surface markers, and washed again in FACS buffer. Intracellular cytokines staining was performed using Fixation/Permeabilization solution kit (BD Biosciences). Cells were fixed and permeabilised using Cytofix/Cytoperm solution for 20 min at 4 °C, followed by a wash in 1x Perm/Wash buffer (diluted 1:10 in distilled H_20). Cells were left in 1x Perm/Wash overnight or immediately stained with antibodies

diluted in 1xPerm/Wash and recognising IFNy and IL-2 (see Table 2-1) for 45 min to 1 h at RT. Cells were protected from light at all incubation steps. The final wash was repeated twice with 1xPerm/Wash, cells were resuspended in FACS buffer, transferred through nitex, and acquired as stated above.

2.6.3 **$CD4^+$ T cell transcription factor staining**

Cells from either *in vitro* co-culture or cell suspension obtained directly from secondary lymphoid organs were washed in DPBS and stained for viability and surface markers as before, followed by a wash in FACS buffer at 400 g for 3 mins. eBioscience Foxp3/Transcription factor staining buffer set (Thermo Fischer) was employed for T-bet expression detection. Fixation/Permeabilization concentrate was diluted in the solution provided and cells fixed for 1 h a RT, followed by a wash in Permeabilization buffer. Cells were then either left in Permeabilization buffer overnight or immediately stained with an antibody for transcription factor T-bet diluted in Permeabilization buffer for 1 h at RT. Cells were protected from light at all incubation steps. The final step involved two washes in 1xPermeabilization buffer, followed by samples resuspended in FACS buffer and transferred through nitex for acquisition.

2.6.4 **Apoptosis marker staining**

After being stained for viability and surface markers, cells were washed twice in FACS buffer and resuspended in Annexin V Binding buffer (Biolegend; diluted 1:10 in dH_20) at $2x10^6$ cells/ml. 50 ul of cell suspension from each sample was transferred into 5 ml FACS tubes and incubated with Pacific Blue-conjugated Annexin V protein (Biolegend) at 1:50 dilution for 15 mins at RT and protected from light. 200 ul of Annexin V Binding buffer was then added to the samples and samples transferred through nitex and acquired as stated above.

2.6.5 Phospho Flow cytometry assays

2.6.5.1 Mouse whole blood stimulation and staining

Mouse whole blood obtained through cardiac puncture was collected into Eppendorfs containing Heparin (1:500) in DPBS and kept on ice. 100 ul of each sample was transferred into FACS tubes together with 100 ul of a cocktail containing antibodies against CD4 and CD44 molecules and IL-6 (100 ng/ml, in DPBS with 5% BSA)(Sigma). Unstimulated samples received antibody cocktail alone. All samples were subsequently incubated at 37 ^{o}C for 15 mins and immediately fixed with 500 ul of pre-warmed Phospflow Lyse/Fix buffer (BD) for 10 min at RT, protected from light. Cells were then spun down, washed twice with cold DPBS, and permeabilised with 1 ml of cold Perm Buffer III (BD) for 30 min on ice, protected from light. Cells were washed twice with cold DPBS and once with cold 5% BSA in DPBS. Intracellular phosphorylated STAT staining was performed with pSTAT3(pY705)-AF647 antibody(BD)(see Table 2-1) prepared at 1:5 in 5% BSA in DPBS. Cells were stained on ice for 30 min protected from light, washed twice with cold 5% BSA in DPBS, transferred through nitex, and resuspended in FACS buffer for acquisition.

2.6.5.2 DC-CD4^{+} T cell co-culture stimulation and staining

BMDCs were stimulated to maturation with LPS as described earlier (Section 2.4.1.) and pulsed with 5 ug/ml of OVA$^{323-339}$ peptide (Sigma) for at least 2 h at 37 ^{o}C 5% CO_2. CD4^{+} T cells were isolated from lymph nodes and spleens of OT-II mice using EasySep kit (Stemcell Technologies) and 1x10^{6} of CD4^{+} T cells were co-cultured with 0.1x10^{6} BMDCs in 24-well culture plates (Corning) overnight in the presence of 10 000 nM of Tofacitinib (base or citrate) (LC Labs) or corresponding concentration of DMSO (Sigma). Following incubation, cells were stimulated with either 100 ng/ml of IL-2 or IL-6 (both-Biolegend) for 15 min at 37 ^{o}C and immediately fixed with 1.25 ml of pre-warmed Phospflow Lyse/Fix

buffer (BD) for 10 min at 37 °C. Samples were then collected into FACS tubes, spun down at 500 g for 5 min with resulting supernatant discarded, and then washed twice in FACS buffer. Cells were treated with FC-receptor block (2.4G hybridoma supernatant) and stained with extracellular antibodies against CD4 and CD44 (see Table 2-1), as described before (see Section 1.6.1), and washed in FACS buffer before being permeabilised with cold Perm Buffer III (BD) for 30 min on ice protected from light. Cells were subsequently washed twice in FACS buffer and stained intracellularly with pSTAT3(pY705)-AF647 and pSTAT5 (pY694)-PE antibodies (both BD)(see Table 2-1) diluted in FACS buffer (at 1:5) for 1 h at RT. Following the final wash, samples were resuspended in FACS buffer for acquisition.

2.7 Enzyme-linked immunosorbent assay (ELISA)

2.7.1 IFNy ELISA

Supernatants were collected from $CD4^+$ T cell-DC co-cultures on day 3, stored at -20°C, and thawed thereafter at RT prior to use. IFNy capture antibody (Thermo Fischer)(see Table 2-3) was diluted in PBS at 1 ug/ml and used to coat 96-well high binding plates (Corning) overnight at 4°C. Plates were then washed twice with PBS/0.05% Tween20 wash buffer and incubated with 200 ul of PBS/1% BSA for 2 h at RT to prevent non-specific binding. Standard was reconstituted in PBS/0.5% BSA and diluted to obtain top standard solution at 800 pg/ml, which was used to set up an 8-point standard curve through serial dilutions. Plates were washed again and 50 ul of standard or sample of appropriate dilution were added to appropriate wells in duplicate, followed by 2 h incubation at RT. After another round of washes in PBS/0.05% Tween20, 50 ul of biotin-labelled antibody (Thermo Fischer)(Table 2-3) was added for 1 h incubation at RT. Plates were washed as before and 50 ul of detection reagent Extravidin-Peroxidase (Sigma) diluted in PBS/0.5% BSA was added to each well and plates incubated for 45 min at RT. Following a final wash, 50 ul of SureBlue TMB substrate (Thermo Fischer) was added to each well and incubated at RT protected from light until

the colour developed in the standard wells. The reaction was terminated by adding 50 ul of 10% sulphuric acid and the resulting light absorbance of each well was measured using Sunrise Microplate reader (Tecan) at 450 nm wavelength.

2.7.2 **OVA ELISA**

Whole blood was collected from RA model mice by cardiac puncture and left in the fridge for several hours to allow the clotting. To obtain the serum, the samples were spun down at 13,000 rpm for 5 mins at 4 ^{o}C and the clear fraction was moved into fresh tubes and spun down again. The resulting serum was aliquoted and stored in fresh tubes at -20^{o}C until use.

For ELISA, high binding 96-well plates (Corning) were coated overnight at 4 ^{o}C with chicken ovalbumin protein (Sigma) dissolved in 0.05 M carbonate buffer. The plates were then washed with PBS/ 0.05% Tween20 wash buffer and blocked with 200 ul of 10% FBS/PBS for 1 h at RT. Plates were subsequently washed, and the serum samples appropriately diluted in wash buffer were added in duplicate. Samples from the HAO-challenged mouse group were added as a positive control, and the negative control contained wild type mouse serum. Doubling dilutions were performed for all samples, starting at an initial 1:100 dilution. Plates were incubated for 2 h at RT allowing a-OVA antibodies from serum to bind to the protein in the wells, followed by another wash. 100 ul of biotin-labelled IgG1 or IgG2a antibody (both BD Pharmigen) (Table 2-3) was added for 1 h incubation at RT. After another round of washes, 100 ul of Extravidin-Peroxidase substrate (Sigma) diluted in wash buffer was added to each well and incubated for 30 min at 37 ^{o}C. Following a final wash, 100 ul of detection reagent SIGMA*FAST* OPD substrate solution (Sigma) was added and incubated, protected from light, until the colour has developed in the positive control wells. The reaction was terminated by adding 50 ul of 10% sulphuric acid and the resulting light absorbance of each well was measured using Sunrise Microplate reader (Tecan) at 492 nm wavelength.

2.7.3 **Collagen II ELISA**

High binding 96-well plates (Corning) were coated overnight at 4 ºC with chicken Type II Collagen (Sigma) in 0.05M carbonate buffer. The plates were then washed with PBS/ 0.05% Tween20 wash buffer and blocked with 200 ul of Animal-free blocker (Vector Laboratories) overnight at 4 ºC. The subsequent steps were the same as for the α-OVA ELISA described above. The positive control samples used to confirm assay efficacy were from the mouse with collagen-induced arthritis (CIA). The HRP-conjugated antibody (Sigma)(Table 2-3) was used to detect total IgG levels. Following antibody incubation and a final wash, SIGMA*FAST* OPD substrate solution (Sigma) was used for signal detection as before, followed by reaction termination with 10% sulphuric acid. The resulting light absorbance of each well was measured as stated above.

2.8 Luminex assay

Following ELISA for cytokines, day 3 co-culture supernatants were assessed by Milliplex Mouse cytokine panel (Merck). Samples were thawed at RT and centrifuged at 10,000 rpmi for 5 min to eliminate cellular debris. Samples were then diluted 1:2 in assay buffer. Cytokine standards were prepared according to the instruction manual provided. Wells were incubated with 200 ul of Wash buffer with agitation for 10 min at RT and the remaining buffer decanted. 25 ul of each control and standard solutions were added to the appropriate wells, while 25 ul of assay buffer used for background wells. 25 ul of culture medium was then added to each of the above wells. 25 ul of diluted samples were plated as appropriate. Beads in each vile were mixed by vortexing and sonication, and 60 ul from each antibody bead vial used to make up a master mix supplemented with Assay buffer. 25 ul of bead mixture was added to all the wells, plate sealed and incubated overnight at 4 ºC protected from light. The plate was then washed twice with agitation with 200 ul of Wash buffer, rested in between washes on a

handheld magnet to settle the beads, and remaining buffer decanted. 25 ul of biotinylated detection antibodies were added for 1 h incubation at RT with agitation, followed by 25 ul of Streptavidin-PE reporter added for another 30 min incubation at RT with agitation. Plate contents were then decanted, and the plate washed as before. For acquisition, 150 ul of sheath fluid was added to all the wells, and the plate incubated for 5 min with agitation. Samples were then assessed using Bio-Rad Luminex 200 plate reader for the presence of IL-2, IL-5, IL-6, IL-10, IL-17A, IFNy, and TNFα. Sample cytokine concentrations were determined from the standard curves constructed by the software for each cytokine. Values below the detectable range were designated as 0 for graphic representation.

2.9 *In vivo* adoptive transfer system for assessment of T cell responses

$CD4^+$ T cells were isolated from lymph nodes and spleens of male OT-II mice using EasySep kit (Stemcell Technologies) and labelled with CFSE as previously described (Section 2.3.1). Cells were then resuspended in sterile DPBS (Sigma) and a total of $1x10^6$ OT-II $CD4^+$ T cells in a final volume of 200 ul were adoptively transferred by intravenous injection (i.v.) into all C56BL/6 male recipient mice. One day following the adoptive cell transfer, recipients were immunized subcutaneously (right footpad) with either 8 ug of LPS from E.*coli* strain 0111: B4 (Sigma) or the same LPS dose together with 25 ug of $OVA^{323-339}$ peptide, both in the final volume of 25 ul. On the day of adoptive transfer, recipient mice received a 25 mg/kg dose of Tofacitinib citrate (LC Labs) prepared in 0.5% Methylcellulose/0.025% Tween20 solution in sterile DPBS (as described in Section 1.1.4.) and administered by oral gavage in a final volume of 100 ul. For the consecutive 3 days, 25 mg/kg of the drug was administered twice daily. Control groups received twice-daily administration of 0.5% DMSO in the same solution and volume as the drug by oral gavage. Three days after the footpad challenge, the recipient mice received a single 25 mg/kg dose of the drug or DMSO and were sacrificed 40 min later. Whole blood was collected from each mouse by

cardiac puncture for assessment of the efficacy of JAK/STAT signalling pathway inhibition by the drug *in vivo* by Phospho Flow cytometry. Popliteal lymph nodes were harvested for analysis of adoptively transferred CD45.1 OT-II $CD4^+$ T cells and their endogenous counterparts by FACS.

2.10 'Breach of tolerance' RA mouse model

2.10.1 Mouse model generation and drug treatment

$CD4^+$ T cells were isolated from lymph nodes and spleens of male OT-II mice using MACS $CD4^+$ T cell isolation kit and polarized over 3 days towards Th1 phenotype(Section 2.3.2). The sample from resulting culture was stained by FACS as described, and the percentage of $V\alpha2^+V\beta5^+$ double-positive cells was used to calculate the total number of Th1 polarized cells obtained. Cells were then resuspended in sterile DPBS and a total of $2x10^6$ polarized Th1 cells in a final volume of 200 ul were injected i.v. into all C56BL/6 male recipient mice. The next day, all recipients were immunized subcutaneously (scruff) with 100 ug of OVA protein in Freund's complete adjuvant (CFA; Sigma), and after another 10 days two treatment groups were challenged by subcutaneous injection in the right and left hind limbs, close to the ankle joints with 100 ug of heat-aggregated OVA (HAO) in 50 ul PBS. The control group received PBS injection instead. Starting one day prior to HAO challenge, mice from one HAO-treated group received Tofacitinib citrate (LC Labs) twice daily (at 25 mg/kg/dose) (prepared as described in Section 2.1.4. and administered by oral gavage) in a final volume of 100 ul. Another HAO-treated group and PBS control group received twice-daily administration of 0.5% DMSO in the same solution and volume as the drug by oral gavage. On day 7 after the HAO challenge, mice received a single 25 mg/kg dose of the drug or DMSO and were sacrificed within 1 h of the last dose.

2.10.2 **Pathology assessment**

On day 3 post-HAO challenge, blood was collected by tail vein bleed for the assessment of phospho-STAT levels by Phospho-Flow cytometry. Starting one day after HAO challenge until the termination of the experiment, the thickness of both hind paws was measured daily using dial caliper (Kroeplin) to monitor the extent of inflammation. After the sacrifice, hind limbs were removed, fixed in 10% neutral-buffer formalin, and decalcified in EDTA solution for 6 weeks. 6 um sections were cut on the sagittal plane and stained with H&E or toluidine blue. Sections were imaged used EVOS FL Auto 2 imaging system (Thermo Fischer). Histological assessment was performed by two observers blinded regarding the treatment group. Each section was scored on the scale 0-3 for cellular infiltration, hyperplasia, and cartilage/bone erosion with a maximal total score of 9 per section. Whole blood was collected by cardiac puncture for assessment of anti-OVA and anti-Collagen II antibody levels in mouse serum. Lastly, popliteal lymph nodes were collected for FACS analysis.

Table 2-1. List of antibodies used for flow cytometric analysis.

Antibody target	Conjugate	Clone	Isotype	Species (host)	Source
CD4	PerCP-Cy5.5	GK1.5	IgG2b, κ	Rat	Biolegend
CD4	eF450	RM4-5	IgG2a, κ	Rat	eBioscience
CD4	BV605	RM4-5	IgG2a, κ	Rat	Biolegend
CD44	APC	IM7	IgG2b, κ	Rat	eBioscience
CD44	BV395	IM7	IgG2b, κ	Rat	BD Biosciences
CD44	PerCP-Cy5.5	IM7	IgG2b, κ	Rat	eBioscience
CD45.1	eF450	A20	IgG2a, κ	Mouse	eBioscience
CD62L	PE	Mel-14	IgG1, κ	Mouse	BD Biosciences
CD69	PE	H1.2F3	IgG1, λ3	Armenian hamster	BD Biosciences
CD69	PE-Cy7	H1.2F3	IgG	Armenian hamster	eBioscience
IL-2	APC	JES6-5H4	IgG2b, κ	Rat	eBioscience
IFNy	PE-Cy7	XMG1.2	IgG1, κ	Rat	Biolegend
T-bet	PE-Cy7	4B10	C	Mouse	eBioscience
pSTAT3 (pY705)	AF647	4/p-STAT3	IgG2a, κ	Mouse	BD Biosciences
pSTAT5 (pY694)	PE	47/pSTAT5 (pY694)	IgG1, κ	Mouse	BD Biosciences

(TCR) Vα2	APC	B20.1	IgG2a, λ	Rat	eBioscience
(TCR) Vß5.1/5.1	FITC	MR9-4	IgG1, κ	Mouse	eBioscience

Table 2-2. List of antibodies used for Western Blot assay.

Antibody target	Clone	Species (host)	Isotype	Source
1º Antibody				
pSTAT3 (Tyr705)	D3A7	Rabbit	N/A	Cell signalling
STAT3	124H6	Mouse	IgG2a	Cell signalling
ß-actin	C4	Mouse	IgG1, κ	Santa-Cruz
2º Antibody				
HRP antibody (anti-mouse)		Donkey	Targeting rabbit IgG	GE Healthcare
HRP antibody(anti-rabbit)		Sheep	Targeting mouse IgG	GE Healthcare

Table 2-3. List of antibodies used for ELISA assays.

Antibody target	Clone	Species (host)	Isotype	Source
IFNy capture antibody	AN-18	Rat	IgG1, κ	eBioscience
IFNy-biotin conjugated	R4-6A2	Rat	IgG1, κ	eBioscience
IgG1	A85-1	Rat	IgG1, κ	BD Biosciences
IgG2a	R19-15	Rat	IgG2a, κ	BD Biosciences
IgG (H+L; total) HRP conjugated	-	Goat	Polyclonal	eBioscience

3 Investigating the effect of JAK3/1 inhibitor Tofacitinib on the DC-CD4^{+} T cell interaction during priming and re-activation stages *in vitro*

Luminex samples were assessed using Biorad reader by Diane Vaughan.

3.1 Introduction

Aberrant $CD4^+$ T cell activation is important in RA development. Activation of self-reactive clones escaping negative selection in the thymus and the periphery and responding to the neo-epitopes generated in RA contribute to the initiation of autoimmune response and self-tolerance breakdown[470,471,472]. Once primed, T cells can migrate to the joint to exert their pathogenic functions through cytokine release and direct cell contact with other immune cells and resident synovial cells[473,474,475,476]. While $CD4^+$ T cells are primed during the disease establishment phase, they also continuously encounter neo-antigens released from the joint tissues undergoing progressive degradation at later stages of RA. Thus, therapeutically interfering with $CD4^+$ T cell initial antigenic encounter may allow one to potentially limit the replenishment of the activated T cell pool and thus, at least partly, control the disease chronicity. Understanding whether and how JAK inhibitor tofacitinib influences $CD4^+$ T cells during priming would provide a deeper insight into its immunomodulatory functions and potentially contribute towards the improvement of the clinical efficacy of the drug.

Alongside the newly primed T cells, the activated/memory effector T cells are equally important in sustaining and perpetuating the chronic autoimmune response. Since the initiation of an autoimmune response is observed in patients up to a decade before the symptoms begin to manifest, by the time the patients get diagnosed and start receiving treatment most of the immune cells and the resident synovial cells are activated[477]. This is evidenced by $CD4^+$ T cells found in the RA patient synovium to exhibit activated phenotype [478,479,480]. In addition, the antigen-experienced lymphocytes have a much lower activation threshold compared to their naïve counterparts and can bypass the requirement for TCR-dependent re-stimulation and undergo bystander activation by a combination of co-stimulation signals and cytokine cues abundant in the inflamed synovial environment[481,482]. Therefore, the secondary stimulation of these activated $CD4^+$ T cells provides another important checkpoint for potential therapeutic targeting and thus should be explored in the context of tofacitinib treatment.

Multiple studies using human lymphocytes from both healthy donors and RA patients have been conducted to determine the effect tofacitinib treatment has on the lymphocyte populations, including the $CD4^+$ T cell compartment. In a study by Maeshima *et al*. $CD4^+$ T cells isolated from peripheral blood mononuclear cells (PMBCs) and synovium of RA patients and re-activated *ex vivo* in the presence of increasing tofacitinib concentrations demonstrated diminished proliferative potential alongside with reduced IFNy and IL-17 cytokine production, both affected in a dose-dependent manner.[306] In another study, healthy individuals subjected to a 4-week treatment with clinically relevant tofacitinib dose demonstrated a profound reduction in all subsets of activated T cell subsets, including $CD4^+$ T cells, which potentially correlated with inhibition of activated T cell proliferation rather than direct effect of the drug on the ability of the cells to undergo activation. By contrast, naïve and central memory $CD4^+$ lymphocyte counts have increased through the treatment period. Further, IFNy-producing $CD4^+$ T cells re-stimulated *ex vivo* in both antigen-specific manner and with plate-bound anti-CD3/CD28 antibodies showed reduced IFNy production levels after tofacitinib treatment. Interestingly, these effects were completely reversed upon drug withdrawal. The authors thereby suggest that tofacitinib preferentially targets activated $CD4^+$ T cell subset proliferation and function while their naïve and central memory counterparts are preserved.[483] Correspondingly, a study by Piscianz *et al*. showed tofacitinib exposure during healthy human PBMC phytohemagglutinin (PHA)-activation *ex vivo* to almost completely abolish cell proliferation and reduce activation, assessed by CD25 expression, as well as inhibited production of several cytokines, including IL-2, IL-17 and IFNy. While the removal of the drug restored cell proliferative ability and capacity for CD25 expression, the production of most cytokines remained supressed.[484] The same group in the later study have confirmed the significant impairment of CD25 expression and IFNy production by PBMCs but detected only a modest effect on cell proliferation.[485]

While these studies overall demonstrate the ability of tofacitinib to inhibit both proliferation and cytokine production in re-activated human $CD4^+$ lymphocytes, the PBMCs utilized contain a combination of naïve, effector and memory $CD4^+$ subsets at different stages of activation, thus making it difficult to pinpoint whether the drug specifically interferes with the priming or reactivation of these

cells. Moreover, the polyclonal nature of the circulating human lymphocytes is not suitable for assessing T cell activation in the antigen-specific manner, which is more physiologically relevant in the context of the disease. The immunomodulatory effects of tofacitinib have also been extensively investigated in multiple rodent models of RA, but a thorough drug assessment in the *in vitro* setting is very limited.[305,310,311,312]. Therefore, in this project I utilize a reductionist approach by employing naïve $CD4^+$ T cells from OT-II transgenic mice, expressing αβTCR specific for chicken ovalbumin peptide 323-339 ($OVA^{323-339}$) and recognised in the context of MHC-II (I-Ab) complex[469]. *In vitro* co-culture of these naïve antigen-specific $CD4^+$ T cells with $OVA^{323-339}$ peptide-presenting murine bone marrow-derived dendritic cells presents an excellent tool for effectively mimicking either T lymphocyte priming or re-activation and individually evaluating the impact of tofacitinib treatment on the outcome of both stages of $CD4^+$ T cell-DC interaction. Importantly, the OT-II $CD4^+$ T cells can be also be effectively employed for adoptive transfer experiments, thus allowing to replicate the *in vitro* experiments into the *in vivo* setting.

Thereby, by employing the above antigen-specific co-culture system, I sought to address the following aims:

- To confirm the inhibition by tofacitinib of the JAK/STAT pathway in the model $CD4^+$ T cell-DC co-culture

- To assess the effect of tofacitinib treatment on the activation status and proliferative capacity of antigen-specific $CD4^+$ T cells during priming

- To address the capacity of $CD4^+$ T lymphocytes to differentiate and produce lineage-specific cytokines upon tofacitinib exposure during priming

- To examine the persistence of $CD4^+$ T cell phenotype acquired during priming upon secondary antigenic challenge

- To investigate the impact of tofacitinib on the previously activated (antigen-experienced) $CD4^+$ T lymphocytes

I hypothesise that inhibition of the signalling through the JAK/STAT pathway by tofacitinib will impact the efficiency of $CD4^+$ T cell-DC primary interaction and lead to alterations in the resulting $CD4^+$ T cell phenotypic and functional profile. I also propose that the acquired $CD4^+$ T cell phenotype might persist upon antigenic re-challenge.

3.2 Results

3.2.1 Tofacitinib (base) effectively inhibits γc and gp130 cytokine signalling by blocking JAK1 and JAK3 enzymatic activity downstream of cytokine receptors *in vitro*

Prior to investigating the effect of tofacitinib on $CD4^+$ T cells, it was crucial to confirm that the drug in my hands had the capacity to block its target molecules and subsequently modulate the downstream signalling cascade. Tofacitinib is a potent inhibitor of both JAK3 and JAK1 kinases, which relay the signals from cytokine receptors by recruiting and phosphorylating different combinations of STAT proteins, depending on the type of the cytokine receptor upstream. Since inhibition of JAK enzymatic activity leads to their inability to phosphorylate STAT proteins, levels of phosphorylated STAT forms in the cells are routinely used to assess JAK activity. Hence, we used the changes in phosphorylated STAT levels to confirm the inhibitory capacity of tofacitinib.

First, I assessed the changes in phosphorylated STAT (pSTAT) levels in bone-marrow derived dendritic cell monoculture. The cells were stimulated with the modified stimulation cocktail developed for the Scottish Nested Arthritis Progression cohort (SNAP). The cohort was formed of patients with newly diagnosed RA who initiated methotrexate monotherapy, with the purpose of establishing the potential cellular signatures predictive of different clinical outcomes (disease progression or remission).[486] The PBMCs of these patients were subjected to deep immunophenotyping, including the measurement of their STAT phosphorylation status following stimulation with a cocktail containing several cytokines, phorbol 12-myristate 13-acetate (PMA), ionomycin, as well as α-CD3 and α-CD28 antibodies (see Chapter 2, Section 2.5.1). The latter antibodies were amended as irrelevant for dendritic cell stimulation. Tofacitinib was used at 2 concentrations-100 nM and 1000 nM- corresponding to approximate maximal and total body exposure concentrations in the whole blood of RA patients achieved after a standard tofacitinib dose of 5 mg twice a day.[344]

DMSO (vehicle) used to dissolve the drug is known to be toxic at high concentrations, therefore I included control samples treated with DMSO at concentrations corresponding to those at final drug dilutions in culture media to ensure the observed changes can be attributed to the direct effect of tofacitinib treatment rather than an above confounding factor.

BMDCs were incubated overnight in the presence of either one of the above concentrations of tofacitinib or vehicle control. After subsequent stimulation with the SNAP cocktail for 15 min, cells were lysed and assessed by Western Blotting for the levels of phosphorylated STAT3, activated by IL-6 signalling through the gp130 receptor (in conjunction with IL-6R). The SNAP cocktail stimulation induced detectable STAT3 activation/phosphorylation levels in non-treated cells, comparable to those in both vehicle-treated groups (Figure 3-1). Exposure to tofacitinib at 100 nM notably inhibited STAT3 phosphorylation in stimulated cells, which appears to be further reduced at higher tofacitinib dose of a 1000 nM, to a level close that of unstimulated cells. Total levels of STAT3 remain unaltered under all conditions, confirming the treatment to specifically prevent STAT3 proteins from being activated through phosphorylation. This result demonstrated the ability of tofacitinib to interfere with the signalling through gp130 cytokine receptor by blocking the enzymatic activity of gp130-associated JAK1 and thereby preventing the downstream STAT3-mediated signal transduction.

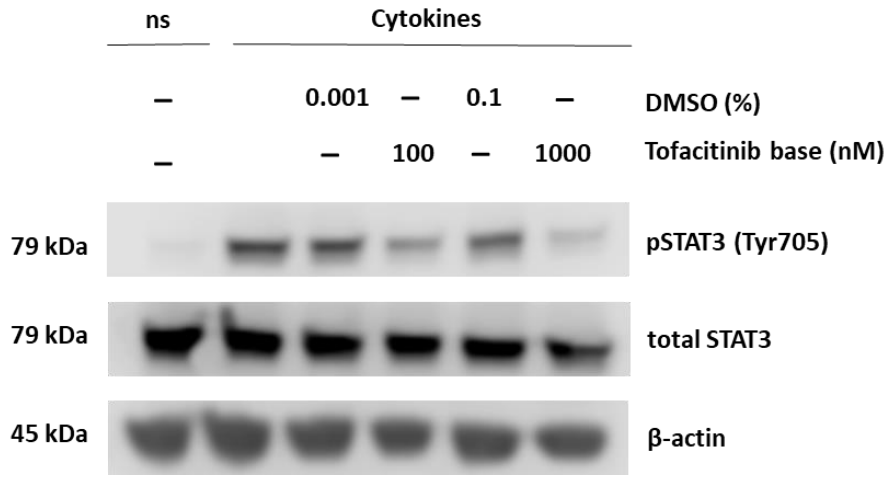


Figure 3-1. JAK inhibitor tofacitinib supresses phosphorylation of STAT3 signal transducer protein in BMDCs in response to cytokine stimulation.

Mature BMDCs were cultured overnight in the presence of either 100 or 1000 nM tofacitinib base (in DMSO) or the vehicle alone and subsequently stimulated with SNAP cocktail (IL-4, IL-6, IFNy and GM-CSF supplemented with PMA/Ionomycin) for 15 min at 37^{o}C. Levels of phosphorylated STAT3 form were assessed by Western blotting. The middle band, representing total levels of both phosphorylated and non-phosphorylated STAT3 forms, was employed as additional loading control. Ns, non-stimulated.

Most of the *in vitro* experiments in my project employ antigen-specific dendritic cell-$CD4^+$ T cell co-culture, therefore the next step was to confirm the capacity of tofacitinib to modulate JAK/STAT pathway signalling in the co-culture setting with no external cytokine input. $CD4^+$ T cells were co-cultured with mature dendritic cells in the presence or absence of $OVA^{323-339}$ peptide and with or without 100 nM of tofacitinib, the dose which in the previous experiment appeared to be sufficient to induce detectable changes in pSTAT3 levels.

In the absence of $OVA^{323-339}$ peptide, OT-II $CD4^+$ T cells did not engage in antigen-specific interaction with dendritic cells, thus overall cells did not actively employ cytokine signalling and exhibit basal levels of phosphorylated STAT3 protein (Figure 3-2). When the peptide was added to the culture, a cross-talk involving IL-6 signalling was established, evidenced by increased levels of pSTAT3, comparable to those in a control sample receiving additional IL-6 stimulation. In the presence of tofacitinib IL-6 signalling in the co-cultured cells was notably diminished, with the levels of pSTAT3 close to the baseline. Thereby, I have confirmed that tofacitinib is able to inhibit the JAK/STAT signalling cascade in the context of antigen-specific $CD4^+$ T cell-dendritic cell co-culture *in vitro*.

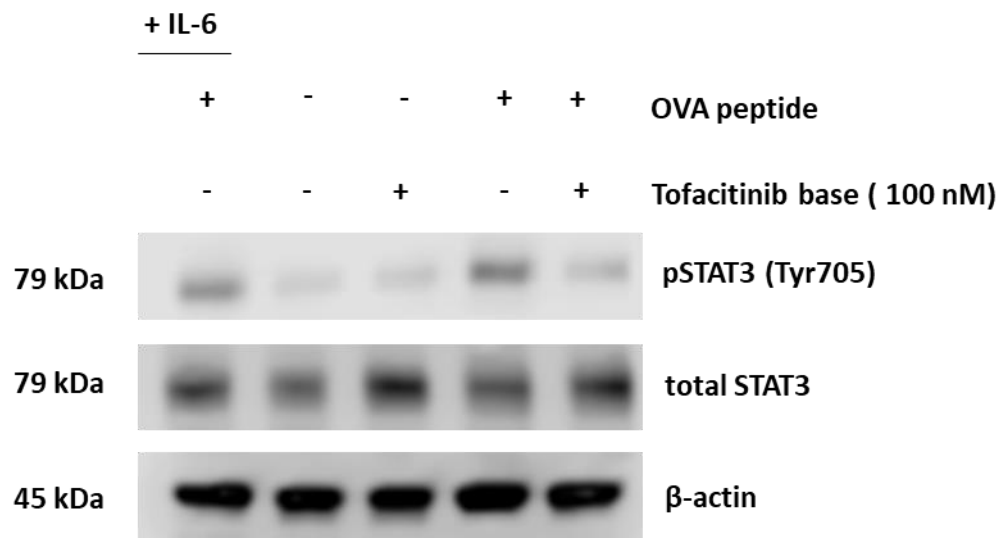


Figure 3-2. Tofacitinib effectively supresses phosphorylation of STAT3 protein in antigen specific $CD4^+$ T cell - DC co-culture.

$1x10^6$ of purified OT-II $CD4^+$ T cells were co-cultured with $0.1x10^6$ of mature bone marrow-derived DCs overnight in the presence or absence of $OVA^{323-339}$ peptide and with either tofacitinib (in DMSO) or vehicle alone. Positive control sample was stimulated with IL-6 (20 ng/ml) for 15 min at 37°C. Levels of phosphorylated STAT3 form were assessed by Western blotting. The middle band representing total levels of both phosphorylated and non-phosphorylated STAT3 forms was employed as additional loading control.

Western blotting proved to be a sensitive enough method to detect the fluctuations in phosphorylation of STAT proteins induced by the drug. However, it required a high number of cells and inconsistent loading control levels have made a number of blots impossible to interpret. In the view of performing *in vivo* experiments later in the project, which would also require to confirm the drug blocking its target pathways but have a very limited yield of cells to assess, a more robust technique was needed. I have thus tested the Phospho-Flow cytometry approach, which allows to simultaneously evaluate levels of several pSTAT proteins even in a small number of cells while distinguishing between different cell populations. I have also utilized the additional stimulation with γc cytokine IL-2, which employs JAK3 and STAT5 for the downstream signalling, to confirm tofacitinib having an impact on its target JAK3 kinase activity.

$CD4^+$ T cells were co-cultured with DCs as previously described and exposed to 10 000 nM of tofacitinib overnight. Samples were subsequently stimulated with either IL-2 or IL-6 for 15 min, immediately fixed and subjected to Phospho-Flow staining for pSTAT5 and pSTAT3 proteins, signalling downstream of IL-2 and IL-6 receptors, respectively. $CD4^+$ T cells presented with their specific antigen in the presence of a vehicle showed a subtle increase in pSTAT3 levels and more substantially elevated pSTAT5 levels in response to respective cytokine stimulation (IL-6 and IL-2) (Figure 3-3). When treated with tofacitinib, $CD4^+$ T cells show impaired ability to receive cytokine signals, as evidenced by lower levels of pSTAT5 relative to vehicle control, and levels of pSTAT3 remaining at the baseline. I have thereby successfully confirmed the Phospho-Flow technique to be an alternative and more effective way to detect changes in different pSTAT protein levels in a cell population of interest *in vitro*. I have also demonstrated that tofacitinib diminishes the function of both of its target kinases JAK1 and JAK3, resulting in impaired signalling downstream of gp130 and γc cytokine receptors, respectively.

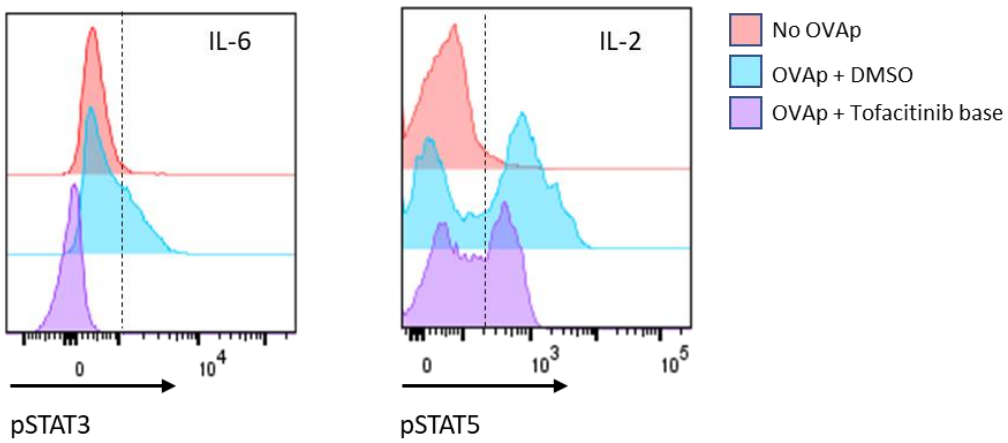


Figure 3-3. Tofacitinib treatment reduces both STAT3 and STAT5 phosphorylation upon cytokine stimulation in CD4$^+$ T cells in co-culture.

Matured bone marrow-derived DCs were incubated with or without 5 ug/ml of OVA$^{323-339}$ peptide for 2 h at 37^{o}C. CD4$^+$ T cells were isolated from OT-II mice lymph nodes and spleens using STEMCELL EasySep kit and cultured with DCs overnight in the presence or absence either 10 000 nM tofacitinib dissolved in DMSO vehicle, vehicle, or no treatment. Cells were subsequently stimulated with either IL-2 or IL-6 (both at 100 ng/ml) for 15 min at 37C, immediately fixed and stained intracellularly for phosphorylated STAT3 and STAT5. Histograms represent levels of pSTAT3 and pSTAT5 in CD4$^+$ T cell populations in response to IL-6 and IL-2 stimulation, respectively. Negative control (pink, no OVAp) received no external stimuli and depicts basal levels of both phospho-proteins in non-activated CD4$^+$ T cells. Dashed lines represent approximate threshold for STAT phosphorylation from the baseline.

3.2.2 Establishment of the optimal OVA$^{323-339}$ peptide dose and effective tofacitinib (base) dose for co-culture experiments

Next, I sought to determine the appropriate concentration of OVA$^{323-339}$ peptide to be used for CD4$^+$ T cell-DC co-culture assays. In our laboratory, a high dose of 5 ug/ml of peptide is routinely used, which provides a strong stimulus to antigen-specific CD4$^+$ T cells via their TCR and might make them less dependent on the cytokine signals during priming. Due to this, it might be harder to detect the potential changes in the T cells responses induced by tofacitinib treatment. Thus OVA$^{323-339}$ peptide dose-response was performed with 10-fold increasing peptide concentrations and the levels of T cell activation marker expression were assessed by flow cytometry following 24 h co-culture with dendritic cells.

CD69 is one of the first markers upregulated in response to antigen-specific T cell activation and was expectedly upregulated in about 60% of cells at the highest peptide dose(Figure 3-4A). CD44 and CD62L are late activation markers with their expression peak at approximately 72 h after T cell priming. Nevertheless, CD44 expression upregulation was detectable and its levels distinguishable between different peptide concentrations (Figure 3-4B). Similarly, loss of CD62L expression upon activation is clearly observed even at this early priming stage (Figure 3-4C). Among all three activation markers the significant change in their expression has occurred in the presence of 0.1 ug/ml of the peptide, which was therefore employed as a sub-optimal peptide dose, alongside with maximal (optimal) dose of 5 ug/ml in the subsequent co-culture experiments.

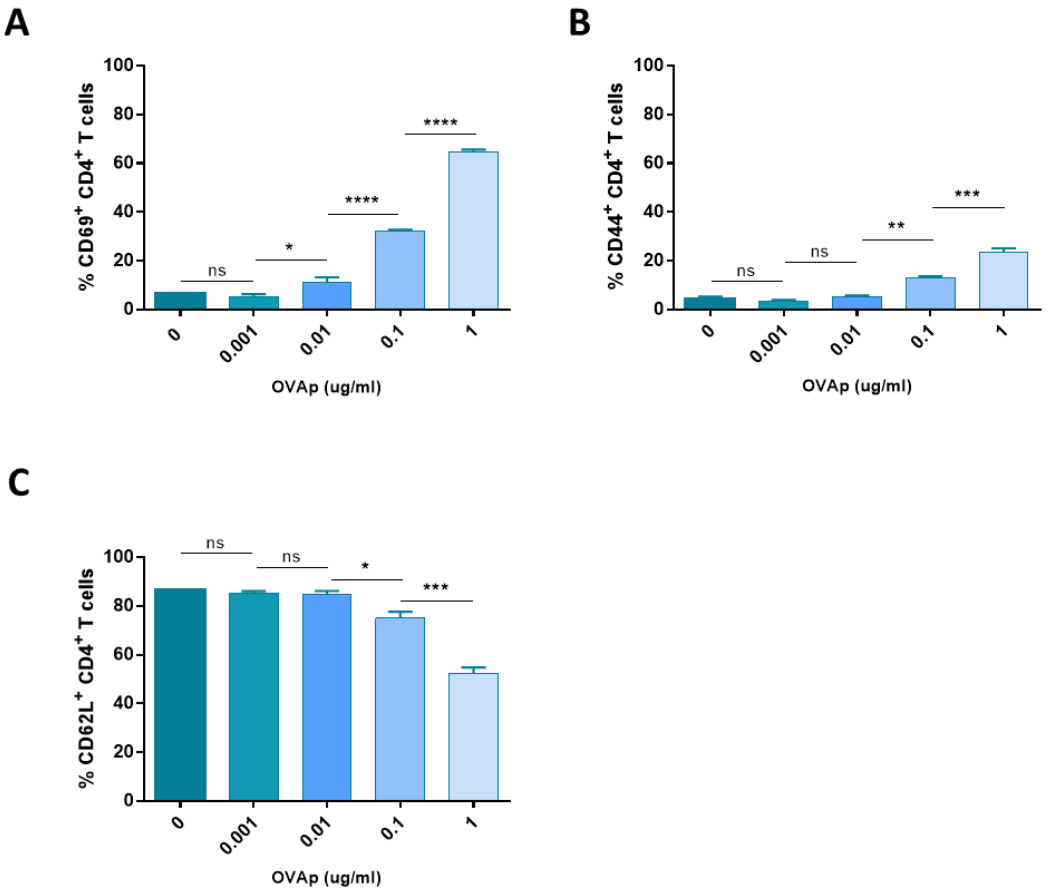


Figure 3-4. The effect of chicken ovalbumin $^{323-339}$ peptide dose range on the activation marker expression in CD4 T^{+} cells in co-culture.

Mature DCs were incubated with increasing concentrations of OVA$^{323-339}$ peptide for 2 h at 37^{o}C and co-cultured for 24 h with CD4^{+} T cells isolated from lymph nodes and spleens of CD45.1 OT-II mice. Samples were then assessed by FACS for the percentage of CD4^{+} T cells expressing early activation marker CD69 (A) and late activation markers CD44 (B) and CD62L (C) when exposed to each of OVA$^{323-339}$ peptide concentrations. Results show the percentage of viable CD4^{+} T cells expressing each of the above activation markers. Data represents mean ± SD for 2 wells per condition. Results represented are from a single experiment. Statistical differences between groups were assessed by performing One-way ANOVA and Turkey's multiple comparison test in GraphPad prism. Ns, non- significant ≥ 0.05, *designates a *p*-value of ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

In the previous chapter section, a tofacitinib dose of 100 nM was shown to be sufficient to notably diminish STAT3 phosphorylation and thereby interfere with the ability of both DCs and $CD4^+$ T cells to receive cytokine signals. In addition to that, a drug dose-response was set-up to determine whether tofacitinib can exhibit a dose-dependent differential effect on $CD4^+$ T cell viability and capacity to express activation markers upon stimulation. $CD4^+$ T cells were activated through incubation with α-CD3 and α-CD28 antibodies, with different tofacitinib concentrations (from 0 to 10 000 nM) added at the start of the assay. After 48 hours cells were assessed by flow cytometry. The analysis demonstrated tofacitinib to have a significant impact on T cell viability when used at 1000 nM and 10 000 nM concentrations (Figure 3-5A). The percentage of T cell expressing activation marker CD69 was slightly reduced starting at 100 nM concentration and became significantly diminished at both 1000 and 10 000 nM concentrations, dropping to approximately 60% and 40%, respectively, when compared to 80% of CD69 positive cells in control group (Figure 3-5B). The percentage of CD44 expressing T cells, on the other hand, was reduced by from 50% to 20% at 100 nM drug concentration and was further diminished at both 1000 nM and 10 000 nM doses to as low as 10% at the highest drug dose (Figure 3-5C). Based on these dose-dependent changes in T cell viability and activation potential, a 100 nM concentration of tofacitinib was selected as the lowest effective dose, while 10 000 nM was chosen as the maximal effective dose. These two drug concentrations were used in all the subsequent co-culture assays *in vitro*.

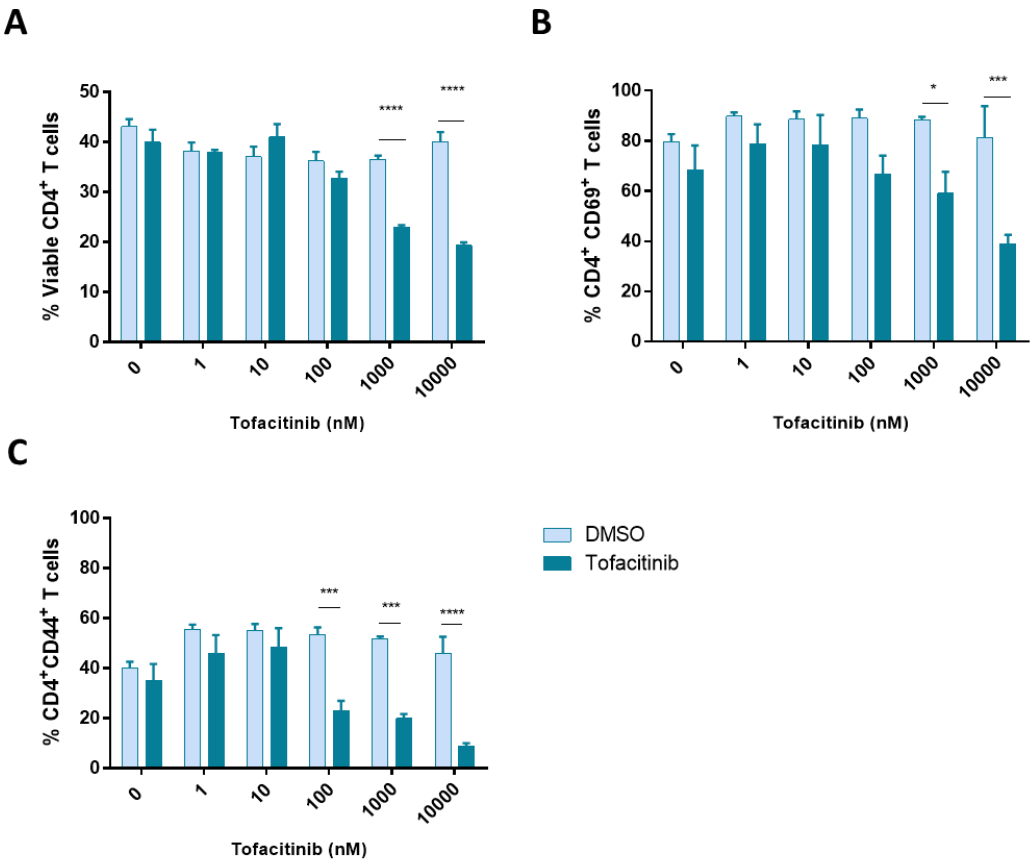


Figure 3-5. Tofacitinib affects CD4⁺ T cell viability and activation marker expression in a dose-dependent manner.

CD4⁺ T cells were isolated from lymph nodes and spleens of CD45.1 OT-II mice using STEMCELL EasySep kit and cultured in the presence of anti-CD3 (1 ug/ml) and anti-CD28 (5 ug/ml) antibodies as an alternative method for T cell activation. CD4⁺ T cells were incubated in the presence of a range of tofacitinib (base) or corresponding vehicle concentrations for 48 h at 37°C and assessed by FACS for the effect of different treatment doses on cell viability (A) and activation marker CD69 (B) and CD44 (C) expression. Cells were gated on total CD4⁺ T cell population (A) or viable CD4⁺ T cell fraction (B and C). Data represents mean ± SD for 3 wells per condition. Statistical differences between groups were assessed by performing Two-way ANOVA and Sidak's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05, *** ≤ 0.001, **** ≤ 0.0001.

3.2.3 Exposure to tofacitinib during priming has no impact on CD4+ T cell activation marker expression and modestly reduces their proliferative potential.

I further sought to investigate if the presence of tofacitinib during CD4+ T cell priming by mature dendritic cells in an antigen-specific manner has an impact on the extent of CD4+ T cell activation. T lymphocyte engagement through TCR/CD3 leads to differential regulation of a range of surface molecules involved in lymphocyte migration and tissue homing *in vivo*, including CD69, CD44, and L-selectin (CD62L).

CD69 is an early lymphocyte activation marker that becomes transiently upregulated following T cells activation and is involved in the regulation of cell ingress and retention in the lymph nodes, as well as egress/migration back to the lymphatic circulation.[487,488,489] CD44 is a late lymphocyte activation marker that promotes T cell interaction with endothelial cells and thereby mediates their recruitment to the site of inflammation[490]. High CD44 expression is retained on the effector and memory cell surface, thus also serving as a permanent marker of antigenic encounter.[491] L-selectin (CD62L) is another late activation marker, which contributes to the coordination of T cell homing to the lymph nodes through high endothelial venules (HEVs).[492] Upon naïve T cell entry to the lymph node and its antigen-specific activation CD62L is terminally shed from the cell surface, allowing the cell to re-enter the lymphatic circulation and migrate to the site of inflammation.[493,494]

Proliferation (clonal expansion) of the antigen-specific T cell population is another key response following naïve T cell priming. After initial TCR engagement leading to extended stable T cell interaction with APC, a T cell undergoes a number of rapid divisions over several days. *In vivo*, this rapid expansion of T cell precursors of a rare antigen specificity underlies the efficiency of adaptive immune response.

To assess the effect of the drug on the T cell ability to undergo antigen-specific activation, CFSE-labelled OT-II T cells were primed as before, with either 0.1 or 5 ug/ml of their cognate peptide presented by mature dendritic cells. After 1

day of co-culture, robust $CD4^+$ T cell activation was confirmed by profound upregulation of CD69 expression at both peptide concentrations, in contrast to the low percentage of CD69-positive cells in the absence of OVA (Figure 3-6A). CD69 expression remained unaffected by the T cell exposure to either 100 nM or 10 000 nM of tofacitinib throughout priming.

Following 3 days of incubation, a majority of $CD4^+$ T cells have proliferated and undergone 2-3 rounds of division (not shown), as indicated by decreased fluorescence intensity of CFSE staining. Treatment with 100 nM of tofacitinib did not impact T cell proliferative ability, however, it was slightly but significantly reduced in the cells treated with higher drug concentration (Figure 3-6B).

Late activation marker assessment showed the expected increase in CD44 expression levels 3 days after T cell priming in the presence of cognate peptide (Figure 3-6C). At suboptimal peptide concentration, CD44 expression was even further increased upon tofacitinib treatment, while at optimal peptide dose CD44 levels in the presence of the compound remained unchanged. Primed T cells also exhibited reduced levels of CD62L expression, and this reduction was slightly but significantly reversed by tofacitinib treatment (Figure 3-6D).

Although the changes in activation marker expression following the drug treatment were statistically significant, they are relatively small and that should be considered when evaluating their biological relevance. I have therefore concluded that exposure of naïve $CD4^+$ T cells to tofacitinib during their antigen-specific priming had no major impact on activation marker expression and only modestly reduced cell proliferative potential.

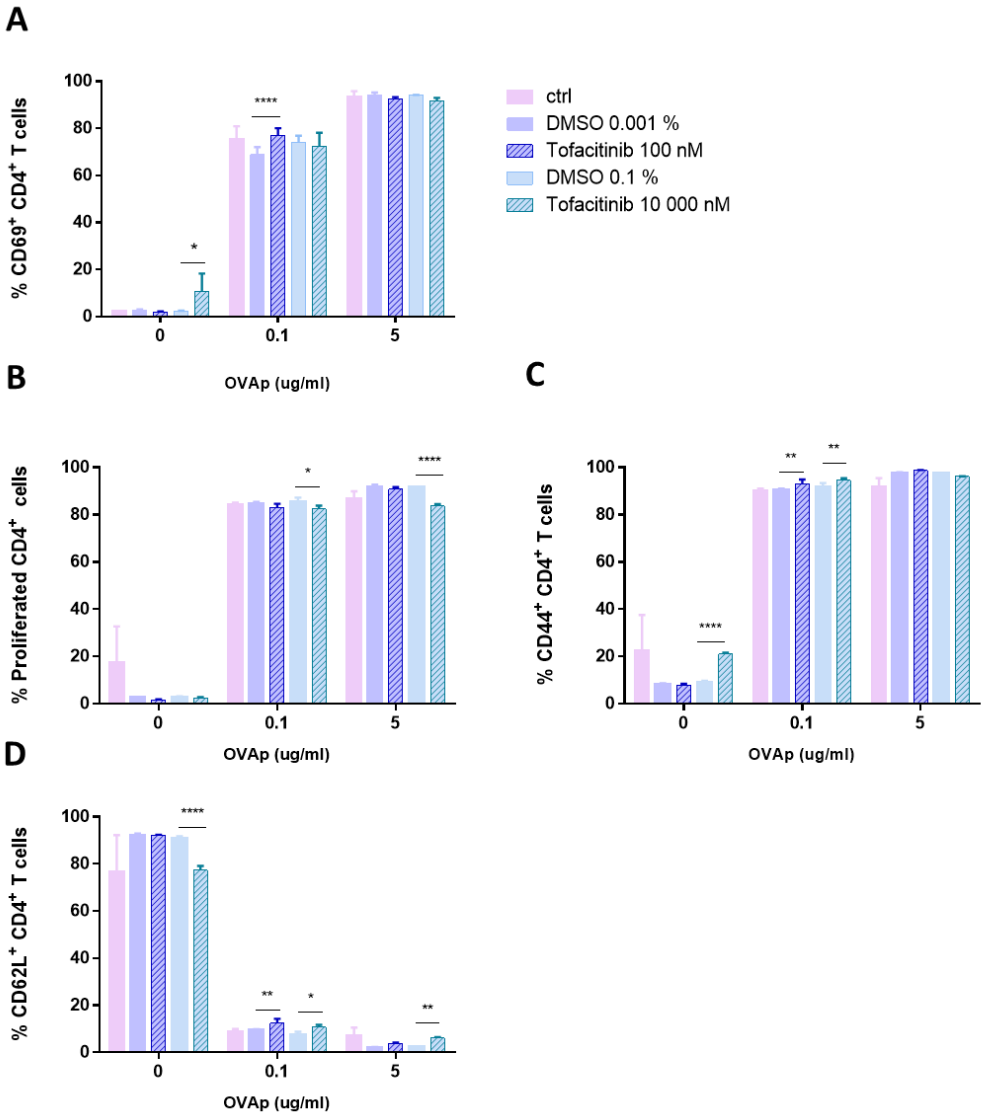


Figure 3-6. Tofacitinib treatment during priming has no effect on CD4⁺ T cell activation marker expression and modestly inhibits their proliferation *in vitro*

CD4⁺ T cells were isolated from lymph nodes and spleens of CD45.1 OT-II mice using STEMCELL EasySep kit and labelled with CFSE dye to monitor cell proliferation. CD4⁺ T cells were then co-cultured with mature DCs in the presence of either 0.1 ug/ml or 5 ug/ml of $OVA^{323-339}$ peptide or without peptide and were treated with either 100 or 10 000 nM of Tofacitinib (base) in DMSO or corresponding vehicle concentrations. After 24 (A) or 72 h (B-D) incubation at 37° C, cells were then stained with a viability dye followed by staining with fluorescent antibodies for CD4, early activation marker CD69 (A) and late activation markers CD44 (C) and CD62L (D). Lymphocytes in graphs (A, C-D) were gated on viable CD4⁺ cells and then analysed for the above activation marker expression. Percentage of proliferated CD4⁺ cells (B) was determined by gating on viable CD4⁺ cells which underwent at least one round of proliferation (designated by reduction in brightness of CFSE fluorescent label). Data represents mean ± SD for 3 wells per condition. Results are representative of 3 independent experiments. Statistical differences between groups were assessed by performing two-way ANOVA and Sidak's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05, ** ≤ 0.01, **** ≤ 0.0001.

3.2.4 Tofacitinib treatment during priming diminishes CD4$^+$ T cell capacity for pro-inflammatory cytokine production *in vitro*

As tofacitinib had no impact on naïve CD4$^+$ T cell activation and small effect on proliferation during priming, I next aimed to assess if the functional capacity of these cells has been compromised. Since antigenic stimulation combined with polarizing signals from APCs (DCs) drive T cell differentiation into different T helper (Th) subsets, I used the production of cytokines specific for main Th subsets as the readout of their function. Following 3 days of OT-II T cell co-culture with OVA peptide-pulsed dendritic cells in the presence or absence of tofacitinib, culture supernatants were collected and analysed for the presence of pro-inflammatory cytokines by Luminex assay.

IL-2 is one of the first mediators secreted by activated CD4$^+$ T cells, which signals in a both autocrine and paracrine fashion and is essential for proliferation and subsequent survival of antigen-specific effector T cells[495,496,497,498]. Starting at 24 h after initial TCR engagement, IL-2 produced by helper T cells initiates autocrine STAT5-dependent negative feedback loop thereby limiting its own production[499]. In our co-culture system, activated T cells secreted high levels of IL-2, which increased in an antigen dose-dependent manner (Figure 3-7A). Treatment with 100 nM of tofacitinib resulted in notably increased IL-2 levels in the supernatant when compared to those in the vehicle-treated samples. IL-2 production was similarly enhanced by higher tofacitinib dose but did not reach statistical significance. Based on the tofacitinib inhibitory mechanism and its ability to reduce STAT5 phosphorylation in response to IL-2 stimulation, demonstrated earlier in this chapter, I propose that the drug interferes with autocrine IL-2 signalling in T cells by blocking JAK3 and JAK1 activation and downstream signalling through STAT5. As a result, a negative feedback loop limiting IL-2 production is disrupted, leading to increased IL-2 generation by activated T cells.

Th1 helper subset, shown to be responsible for arthritis development in both mice and humans, produces its signature cytokine IFNγ upon differentiation, alongside TNFα and IL-2. Tofacitinib was able to suppress IFNγ production by activated T cells to the levels beyond detectable by the assay, suggesting the ability of the drug to interfere with T cell polarization towards the Th1 subtype (Figure 3-7B). While the initial trigger for Th1 polarization is IL-12 provided by APCs (DCs), the resulting IFNγ produced was demonstrated to engage in autocrine signalling to promote T-bet expression, which in turn facilitates further IFNγ production[500]. Since signalling through IFNγ receptor requires STAT1 activation, tofacitinib might act by inhibiting STAT1 phosphorylation and thereby inhibiting this self-enhancing loop of IFNγ production.

Production of TNFα, another Th1-specific cytokine, was also reduced upon exposure to high drug concentrations when compared to vehicle treated cells (Figure 3-7C). However, TNFα production was affected to a lesser degree than IFNγ and the changes seen could be secondary to the direct drug interference with Th1 differentiation and IFNγ-driven positive feedback loop signalling abrogation.

IL-17 producing Th17 helper cells is another important subset mediating inflammation and joint destruction in murine RA models, while in patients Th17 frequencies and IL-17 levels are associated with systemic disease activity[501,502]. Detection of IL-17A in co-culture supernatants from T cells receiving antigenic stimulation suggests some of those cells might have acquired a Th17 phenotype (Figure 3-7D). Their polarization in this *in vitro* setting might be driven by IL-6 and TGFß secreted by mature dendritic cells in combination with IL-21 produced by T cells in response to IL-6 stimulation[503]. Thus, reduction in IL-17A levels in the presence of high drug dose (Figure 3-7D) could be explained by tofacitinib interfering with STAT3-dependent IL-6 and IL-21 polarizing signals and thereby impairing Th17 differentiation.

$CD4^+$ T cell differentiation towards the Th2 subtype, similarly to Th17, also requires an initial specification signal from IL-6. STAT3- dependent IL-6 signal promotes T cells to secrete IL-4, which then acts in an autocrine manner for further differentiation towards Th2 lineage[504]. It was therefore expected for JAK

inhibitor to impair IL-6 driven Th2 polarization, which was evidenced by significantly diminished IL-5 levels in the presence of the drug (Figure 3-7E).

I also assessed the supernatants for IL-6 production, which in this co-culture would be secreted by dendritic cells upon the interaction of CD40 on their surface with its ligand CD40L on activated T cells. Indeed, the increase in IL-6 production is observed in the presence of T cell cognate antigen and is antigen dose-dependent (Figure 3-7F). However, tofacitinib treatment had no effect on IL-6 cytokine levels. This result confirms that the impaired differentiation into Th subsets, which require IL-6 contribution, is a result of the drug interference with cytokine signalling pathway rather than the scarcity of polarizing cytokine in the culture.

Finally, I addressed the impact of tofacitinib on polarization towards the regulatory T cell (Treg) subtype, characterised by the production of IL-10 cytokine and the ability, among others, to suppress Th effector responses. IL-2 signalling (through IL-2Rβ/STAT5 axis) was shown to be a pre-requisite for inducing Treg transcription factor Foxp3 expression[505], suggesting the potential of tofacitinib to also interfere with Treg polarization. However, Treg secreted IL-10 levels upon drug exposure did not decrease, and even increased with the optimal antigen and high drug dose combination (Figure 3-7G). To our knowledge, no studies to date have assessed the capacity of tofacitinib to affect naïve cell differentiation into the Treg subtype. Interestingly, fully differentiated $CD4^{+}CD25^{bright}Foxp3^{+}$ Treg cells from transplant patients receiving tofacitinib treatment appeared to preserve their immunoregulatory function, and their ability to receive IL-2 signals, in contrast to effector T cells, was only partially inhibited by the drug[506]. It would, therefore, be interesting to investigate in more detail whether the drug has differential impact on initial differentiation into helper and regulatory T cell lineages, and if under specific Treg-polarizing conditions naïve T cells are less susceptible to inhibitory drug effect.

Taken together, data from the Luminex assay suggests tofacitinib to diminish the ability of naïve $CD4^{+}$ T cells, primed by their cognate antigen *in vitro*, to undergo differentiation towards Th1, Th2, and Th17 effector subsets. In turn, this results in reduced capacity for their signature pro-inflammatory cytokine

production. In contrast, T cell ability to polarize into the Treg subset is preserved.

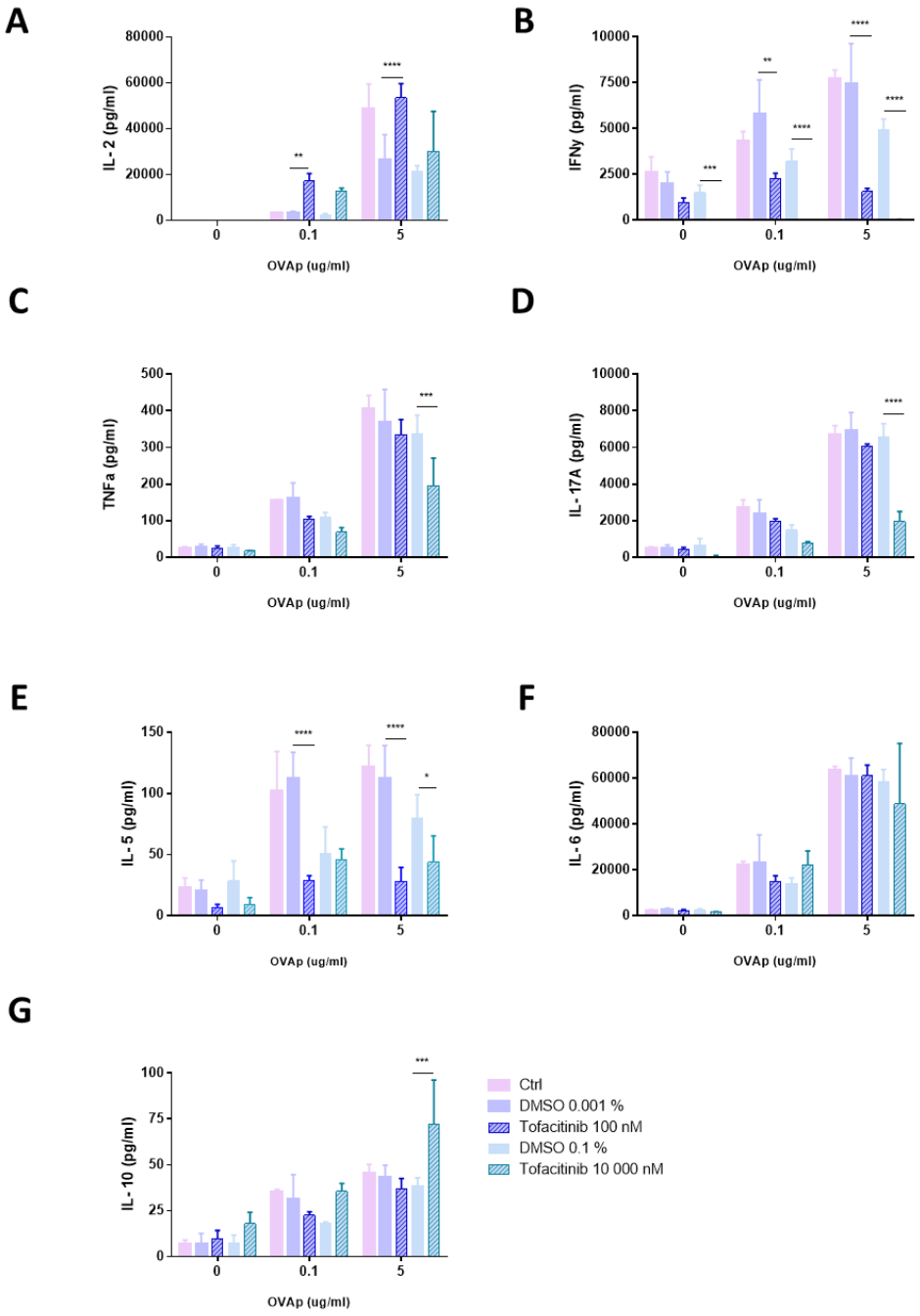


Figure 3-7. Tofacitinib treatment impairs pro-inflammatory cytokine production by CD4⁺ T cells upon priming *in vitro*.

CD4⁺ T cells were isolated from lymph nodes and spleens of CD45.1 OT-II mice using STEMCELL EasySep kit and co-cultured with mature DCs in the presence of either 0.1 ug/ml or 5 ug/ml of $OVA^{323-339}$ peptide or without peptide, and were treated with either 100 or 10 000 nM of Tofacitinib (base) in DMSO or corresponding vehicle concentrations. Supernatants were collected after 3 days of co-culture and analysed by Luminex for the presence of a number of pro-inflammatory cytokines implicated in RA pathogenesis. Graphs depict mean concentrations ± SD of (A) IL-2, (B) IFNy, (C) TNFα, (D) IL-17A and (E) IL-5, (F) IL-6 and (G) IL-10. n=3 wells for each condition. Data represents results from a single experiment. Statistical differences between groups were assessed by performing two-way ANOVA and Sidak's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

I was concerned with the known ability of the drug to induce cell death at high concentrations, which could contribute to the effect seen in the Luminex assay. To address this possibility, a viability assay was performed. OT-II T cells were co-cultured with DCs as before and following 24 h of tofacitinib exposure T cells were assessed for their viability and the presence of phosphatidylserine on their surface as a marker of early apoptosis. The percentage of viable cells remained unaltered in the presence of 100 nM of tofacitinib but have significantly decreased at higher drug concentration (Figure 3-8A). The same T cell population was then assessed for the signs of apoptosis as one of the possible reasons for reduction in the proportion of viable cells. The percentage of apoptotic cells, defined by exclusion of viability dye in combination with Annexin V protein binding to phosphatidylserine on their surface, remained similarly unaffected by low drug dose and have slightly, but significantly increased at high drug concentration (Figure 3-8B). These results suggest that the reduction in cytokine production observed in Luminex assay is partly due to tofacitinib compromising T cell survival. However, the production of some cytokines, in particular IFNy, was almost completely inhibited by the drug and cannot be solely explained by moderate loss of viable cytokine-secreting T cells.

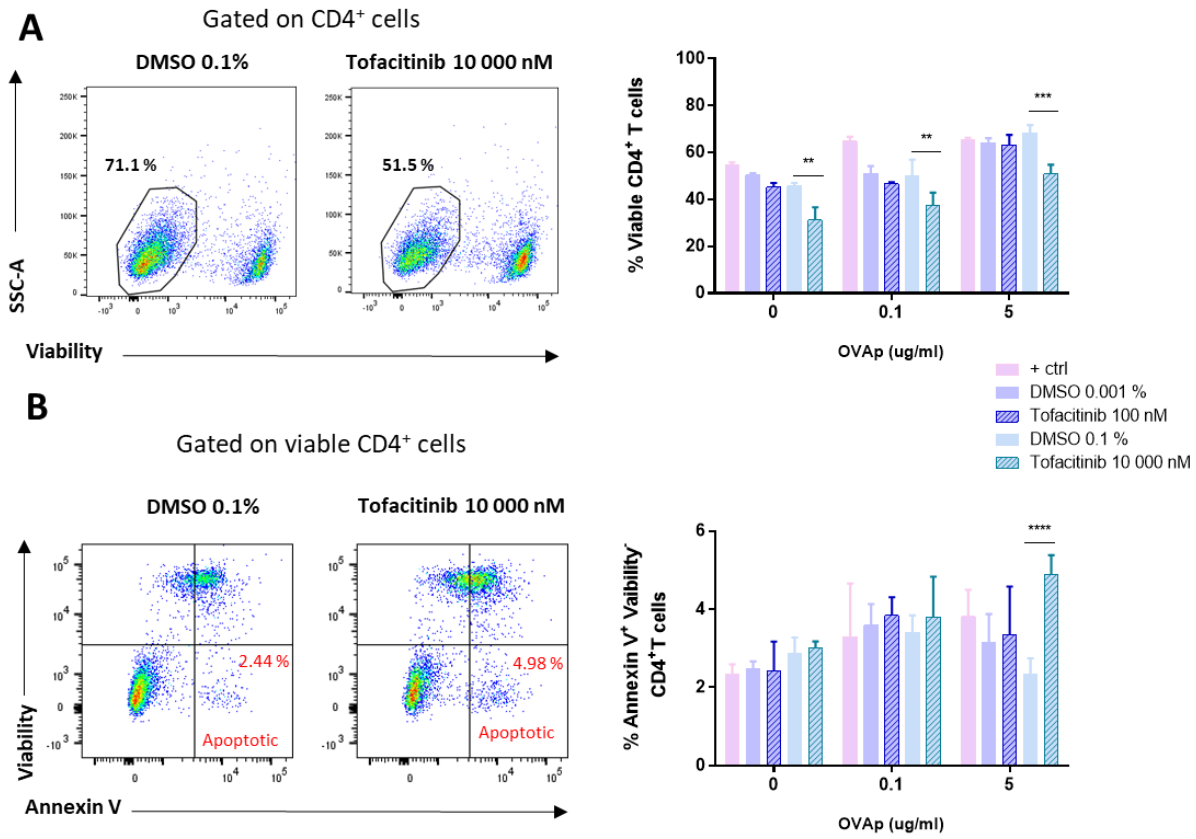


Figure 3-8. Tofacitinib reduces CD4⁺ T cell viability at high concentrations by promoting apoptosis.

CD4⁺ T cells were isolated from lymph nodes and spleens of CD45.1 OT-II mice using STEMCELL EasySep kit and co-cultured with mature DCs in the presence of either 0.1 ug/ml or 5 ug/ml of $OVA^{323-339}$ peptide or without peptide, and were treated with either 100 or 10 000 nM of Tofacitinib (base) in DMSO or corresponding vehicle concentrations. Control samples (in pink) were left untreated. Cells were incubated for 24 hours at 37°C 5% CO_2 and then stained by FACS for viability and CD4⁺ marker expression. Subsequently, cells were incubated with fluorochrome-conjugated Annexin V protein which binds to externalised phosphatidylserine residues on the cells undergoing apoptosis. Lymphocytes were gated on CD4⁺ cells and those negative for viability dye were deemed viable (A). Cells gated as CD4⁺ were further gated for viability against Annexin V, with viability-negative Annexin V-positive population representing early apoptotic cells (B). Representative scatter plots show samples stimulated with 5 ug/ml of the peptide. Graphs depict mean percentages ± SD of viable (A) and apoptotic (B) CD4⁺ T cells from one experiment, n=3 wells for each condition. Statistical differences between groups were assessed by performing two-way ANOVA and Sidak's multiple comparison test in GraphPad prism. ** designates a *p*-value of ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

I therefore decided to investigate the changes in the capacity of viable $CD4^+$ T cells to produce cytokines after exposure to tofacitinib upon their priming. IFNy was selected for further assessment as the cytokine most affected by the drug, alongside with IL-2 as a homeostatic T cell cytokine, signalling of which was evidently inhibited and production enhanced by tofacitinib. In addition, while changes observed in the supernatant could be attributed to the whole cell population, the assessment of T cell capacity to produce cytokines would allow to confirm the intrinsic changes induced in individual cells.

OT-II $CD4^+$ T cells were primed with OVA^{323-33} peptide by mature dendritic cells for 3 days in the presence or absence of tofacitinib, with subsequent PMA/Ionomycin stimulation in the presence of Brefeldin A for 4 h at 37°C. The latter treatment induces intracellular cytokine production by T cells while retaining the cytokines within the cell, thus allowing to assess the potential for cytokine production of an individual cells. While the untreated and vehicle-treated T cells notably increased IFNy production upon priming, the IFNy levels in their drug-treated counterparts remained as low as those of non-activated cells (Figure 3-9A). As expected, a large proportion of the activated T cells produced IL-2 when left untreated, and tofacitinib exposure further increased the percentage of IL-2-positive cells, with this change being significant at an optimal peptide concentration (Figure 3-9B). In addition, an ELISA assay was performed on the day 3 co-culture supernatants, and the dramatic impairment in drug-treated T cell capacity for IFNy production observed in this assay (Figure 3-9C) replicated the results obtained by Luminex and intracellular FACS staining.

Exposure to tofacitinib during priming was therefore demonstrated to induce intrinsic changes in antigen-specific $CD4^+$ T cells, resulting in their reduced ability to produce Th1 lineage-specific cytokine IFNy.

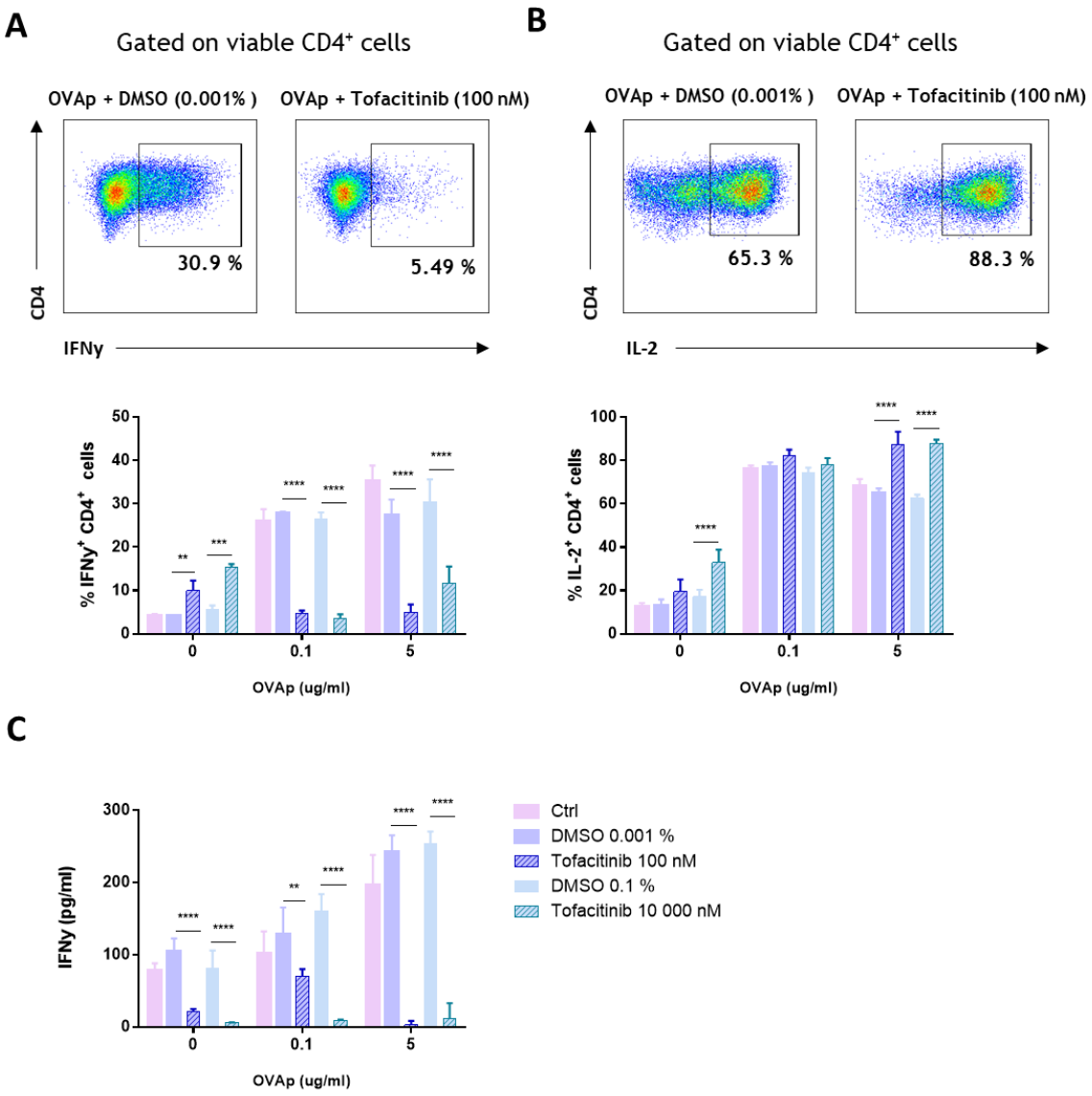


Figure 3-9. Exposure to tofacitinib during priming reduces the capacity of CD4⁺ T cells for IFNy and IL-2 production.

CD4⁺ T cells were isolated from lymph nodes and spleens of CD45.1 OT-II mice using STEMCELL EasySep kit and co-cultured with mature DCs in the presence of either 0.1 ug/ml or 5 ug/ml of $OVA^{323-339}$ peptide or without peptide, and were treated with either 100 or 10 000 nM of Tofacitinib (base) in DMSO or corresponding vehicle concentrations. Control samples (in pink) were left untreated. After 3 days, cells were stimulated with PMA (10 ng/ml) and Ionomycin (500 ng/ml) in the presence of Brefeldin A for 4 h at 37°C 5% CO_2. Thereafter, cells were stained by FACS for viability and CD4⁺ marker expression, followed by fixation/permeabilization step and fluorescent labelling of intracellular IFNy and IL-2 cytokines. Lymphocytes were gated on viable CD4⁺ cells, with representative scatter plots depicting the difference in the percentage of IFNy (A) and IL-2 (B) producers between vehicle- and drug-treated samples, when cultured with 5 ug/ml of the peptide. Graphs shows mean percentages ± SD of viable CD4⁺ T cells producing IFNy (A) and IL-2 (B) upon stimulation. Results are representative of 3 independent experiments.

Supernatants were collected prior to PMA/Ionomycin stimulation and assessed by ELISA for IFNy production in the co-culture over 3 days (C). The graph depicts mean concentration ± SD of IFNy, n=3 wells for each condition. Results are representative of a single experiment. Statistical differences between groups were assessed by performing two-way ANOVA and Sidak's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

3.2.5 Tofacitinib administration upon priming impairs CD4$^+$ T cell differentiation towards Th1 subtype

After repeatedly demonstrating an abolishment of IFNy production upon drug exposure during CD4$^+$ T cell priming, I sought to establish if this is a result of underlying impairment in cell differentiation on transcription factor level. T-box expressed in T cells (T-bet) belongs to the T-box transcription factor family and is found to be indispensable for lineage commitment in CD4$^+$ T cells towards Th1 subset. T-bet has a capacity to promote its own transcription as well as induce chromatin remodelling of *Ifng* locus, thereby regulating the expression of Th1 signature cytokine IFNy.[507,508] Therefore, I examined the effect of tofacitinib treatment on T-bet expression upon priming. In the control samples in the absence of any treatment (pink bars) T-bet expression in CD4$^+$ T cells was induced with antigen addition in a dose-dependent manner, with the vehicle-treated samples closely replicating transcription factor levels similar to those of untreated controls (Figure 3-10B). However, the addition of tofacitinib to the culture during priming profoundly inhibited T cell ability for T-bet expression, retaining transcription factor levels close to the baseline (Figure 3-10A,B). Unexpectedly high T-bet levels were detected in the absence of the peptide stimulation in drug-treated groups (Figure 3-10B, shaded bars). Cells from some of those samples have proliferated and undergone activation, as evidenced by CFSE dilution and upregulated CD44 expression (data not shown). However, as tofacitinib was earlier confirmed to have no pronounced effect on either of those phenotypic changes, the activation of the cells in the no-peptide group along with T-bet expression was most likely experimental artefacts and not the direct result of the drug exposure.

These results suggest the ability of tofacitinib to interfere with CD4$^+$ differentiation towards Th1 lineage by abolishing essential transcription factor T-bet expression.

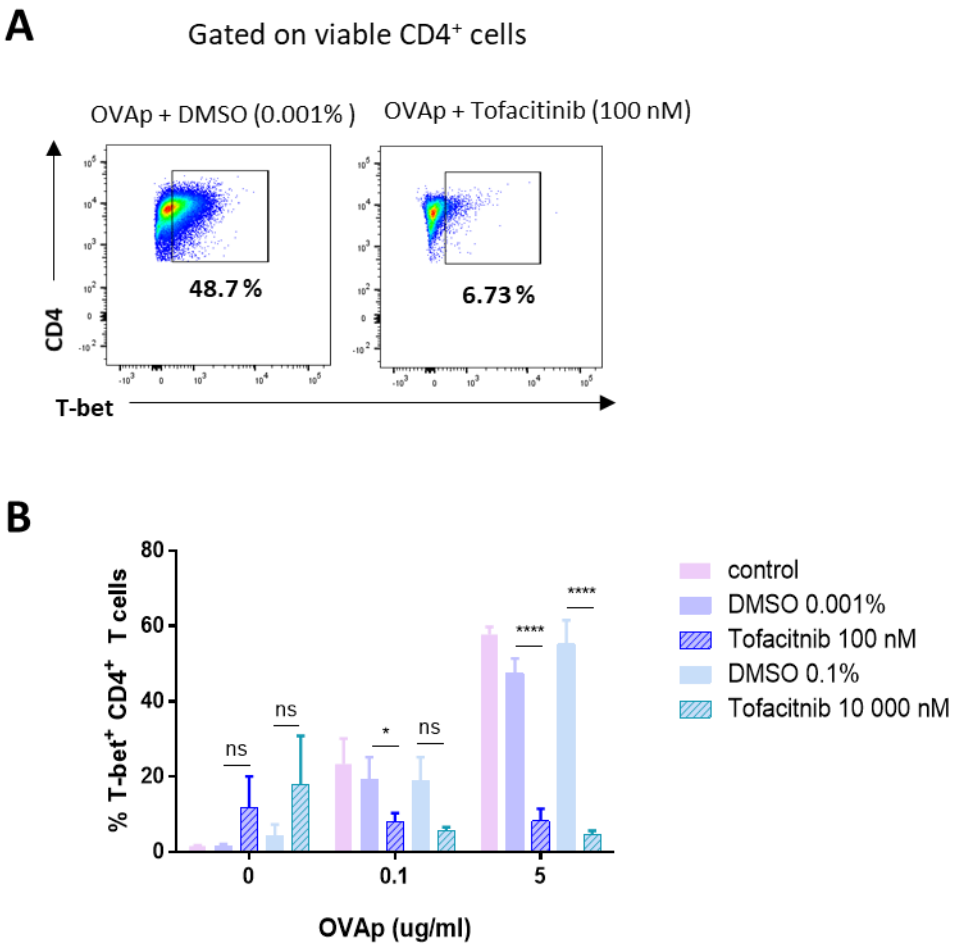


Figure 3-10. Tofacitinib treatment during priming impairs CD4⁺ T cell differentiation towards Th1 phenotype.

CD4⁺ T cells were isolated from lymph nodes and spleens of CD45.1 OT-II mice using STEMCELL EasySep kit and co-cultured with mature DCs in the presence of either 0.1 ug/ml or 5 ug/ml of $OVA^{323-339}$ peptide or without peptide, and were treated with either 100 or 10 000 nM of Tofacitinib (base) in DMSO or corresponding vehicle concentrations. Control samples (in pink) were left untreated. After a 3-day co-culture, cells were stained for FACS for viability and CD4⁺ marker expression, followed by permeabilization/fixation step using Foxp3/Transcription factor staining buffer set and intracellular staining for transcription factor T-bet detection. Lymphocytes were gated on viable CD4⁺ cells, with representative scatter plots depicting the difference in the percentage of cells expressing T-bet in the vehicle- and drug-treated samples (A) when cultured with 5 ug/ml of the peptide. (B) Graphs shows mean percentages ± SD of viable CD4⁺ T cells expressing T-bet, n=3 wells for each condition. The results are representative of 2 individual experiments. Statistical differences between groups were assessed by performing two-way ANOVA and Sidak's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

3.2.6 Impaired T-bet expression and IFNy production following CD4$^+$ T cell exposure to tofacitinib during priming persists upon secondary antigenic challenge

After establishing the ability of tofacitinib to interfere with Th1 cell differentiation during priming, it was interesting to investigate if this effect is maintained after secondary antigen exposure when the drug is no longer present. To address this question, naive OT-II CD4$^+$ T cells were primed with OVA peptide-pulsed DCs for 3 days in the presence of a vehicle or 100 nM Tofacitinib, then thoroughly washed to remove the drug from the media and rested in the media supplemented with IL-2 to support their survival. After being rested for 3 days, activated T cells were labelled with CFSE and either re-challenged with the same cognate peptide dose or left unstimulated for the final 3 days (Figure 3-11A). To confirm the efficiency of secondary antigenic challenge, T cells were assessed for proliferation and activation marker expression. After re-activation, approximately 60% of T cells have proliferated compared to only 10% of non-stimulated cells in both treatment groups, with tofacitinib exposure exhibiting no effect on proliferation (Figure 3-11B). Similarly, CD69, although an early activation marker, was upregulated upon re-activation to 80% from 60% in the T cells receiving no secondary challenge, with the drug having no impact on marker expression levels (Figure 3-11C).

Since T cell re-activation with cognate peptide has proven successful, the transcriptional and functional profile of these cells was examined next. Interestingly, T cells exposed to tofacitinib during priming expressed significantly lower levels of T-bet transcription factor upon re-activation when compared to vehicle-treated counterparts (Figure 3-11D). In addition, T cells primed in the presence of the drug and subsequently re-activated exhibited a reduced capacity for IFNy production upon PMA/Ionomycin treatment, with their IFNy levels comparable to those in the absence of secondary stimulation (Figure 3-11E). Interestingly, T cells that were only primed but did not receive secondary peptide stimulation also retained lower IFNy production levels if exposed to the drug during initial stimulation, which is consistent with the changes in cytokine production detected upon T cell priming.

Together, this data suggests that the diminished T-bet expression achieved with tofacitinib treatment during priming and the resulting impairment in $CD4^+$ T cell capacity to produce IFNy both persist upon secondary antigenic stimulation. In other words, once the drug compromises differentiation into Th1 subset during priming, this compromised cell state remains imprinted, possibly on epigenetic level, upon the subsequent re-activation and is manifested by diminished IFNy production by those T cells.

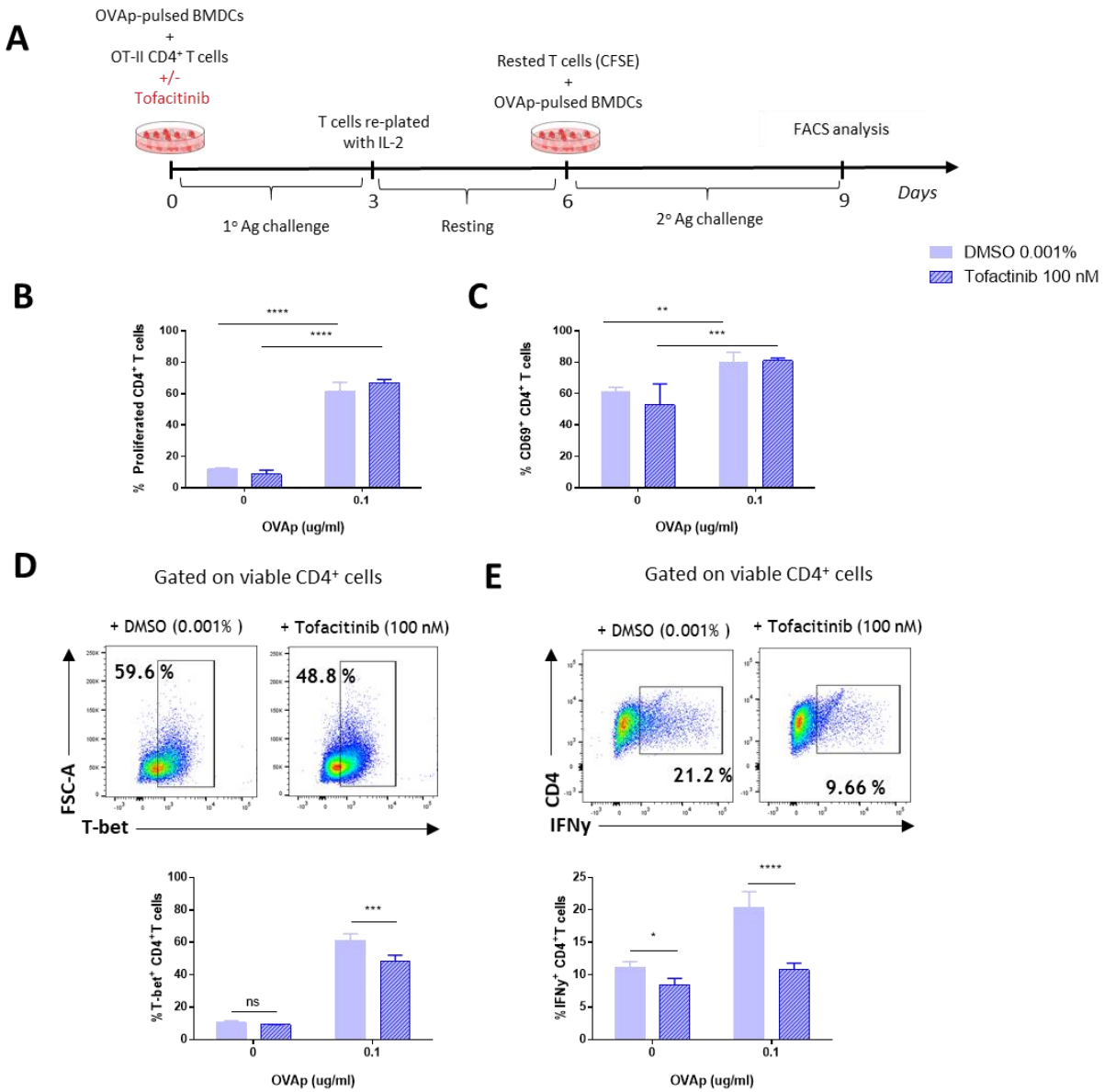


Figure 3-11. Impaired T-bet expression and IFNy production following CD4⁺ T cell exposure to tofacitinib during priming persists upon secondary antigen challenge

(A) CD4⁺ T cells were isolated from lymph nodes and spleens of CD45.1 OT-II mice using STEMCELL EasySep kit and co-cultured with mature DCs in the presence of 0.1 ug/ml of $OVA^{323-339}$ peptide, and were treated with either 100 nM of Tofacitinib (base) or 0.001% of DMSO. After 3 days, cells were washed, re-plated and rested in the presence of IL-2 (1 ng/ml) for another 3 days. Following this, T cells were labelled with CFSE and stimulated with fresh DCs, either untreated or pulsed with $OVA^{323-339}$ peptide, for the final 3 days. Half of the cells were subjected to PMA/Ionomycin treatment in the presence of Brefeldin A, and then stained by FACS for intracellular IFNy production using BD Fixation/Permeabilization kit. Another half of the cells were directly stained for T-bet expression using Foxp3/Transcription factor staining buffer set. Lymphocytes were gated on viable CD4⁺ T cells and analysed for the percentage of cells which have proliferated (B), CD69 (C) and T-bet expression (D), and IFNy production (E). Representative scatter plots depict the percentage of T-bet expression (D) and IFNy producing cells (E) in samples treated with vehicle or 100 nM of Tofacitinib and receiving the secondary antigenic challenge. Graphs shows mean percentages ± SD of viable CD4⁺ T cells expressing the respective markers. Results are representative of 2 individual experiments assessing T-bet expression and 3 separate experiments assessing IFNy production. Statistical differences between groups were assessed by performing two-way ANOVA and Sidak's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

3.2.7 Antigen-experienced CD4$^+$ T cells exhibit diminished T-bet expression following re-activation in the presence of tofacitinib, but retain their capacity for IFNy production

Next, I went on to investigate whether tofacitinib has the capacity to affect previously activated cells when administered during secondary antigenic challenge. This *in vitro* scenario might be particularly relevant if applied in the clinical context, where RA patients receive tofacitinib at progressive stages of the disease and therefore present with a high proportion of CD4$^+$ T cells which are already activated.

CD4$^+$ T cells were primed for 3 days and then rested in the presence of IL-2, as described in the section above (Section 3.2.6.). The antigen-experienced CFSE-labelled T cells were then re-challenged in the presence of vehicle or tofacitinib for another 3 days (Figure 3-12A). Two individual co-cultures were performed to assess transcriptional profile and IFNy production of the resulting CD4$^+$ T cells. To ensure that the T cells were re-stimulated effectively, their proliferative capacity and CD69 marker expression were examined. T cells have proliferated efficiently and significantly when compared to the cells in the absence of secondary stimulation (Figure 3-12B). Interestingly, tofacitinib treatment notably increased the percentage of proliferating cells at both drug doses, which has not been observed in other experiments. Early marker CD69, though slightly upregulated upon T cell re-stimulation, was expressed at relatively low levels (Figure 3-12C), which could be explained by its transient expression prominent at approximately 24 h after cell activation and significantly declining by 72 h post-activation[498]. The CD69 levels appeared to be even lower in the T cells re-activated in the presence of the drug. This effect of tofacitinib on CD69 expression was not detected previously, in either priming or re-activation co-culture experiments. One possible explanation could be that since more of the drug-treated T-cells have proliferated, they may be at the further/later stages of their activation and hence their CD69 expression might be already in decline.

The assessment of T cell differentiation status upon re-challenge showed the significant upregulation of T-bet levels relative to control cells receiving no secondary stimulation. Exposure to tofacitinib during secondary challenge notably diminished T-bet expression in activated T cells at both antigen concentrations (Figure 3-12D). In a separate experiment, a similar percentage of T cells producing IFNy upon re-activation was detected, regardless of the treatment administered (Figure 3-12E). In fact, the percentage of IFNy-producing cells was slightly increased in the group treated with 100 nM tofacitinib during re-challenge.

These results convincingly demonstrate that while tofacitinib supresses Th1-specific transcription factor expression in antigen-experienced cells upon re-activation, their capacity for cytokine production might be at least partly independent of the T-bet levels and remains unchanged following the drug treatment.

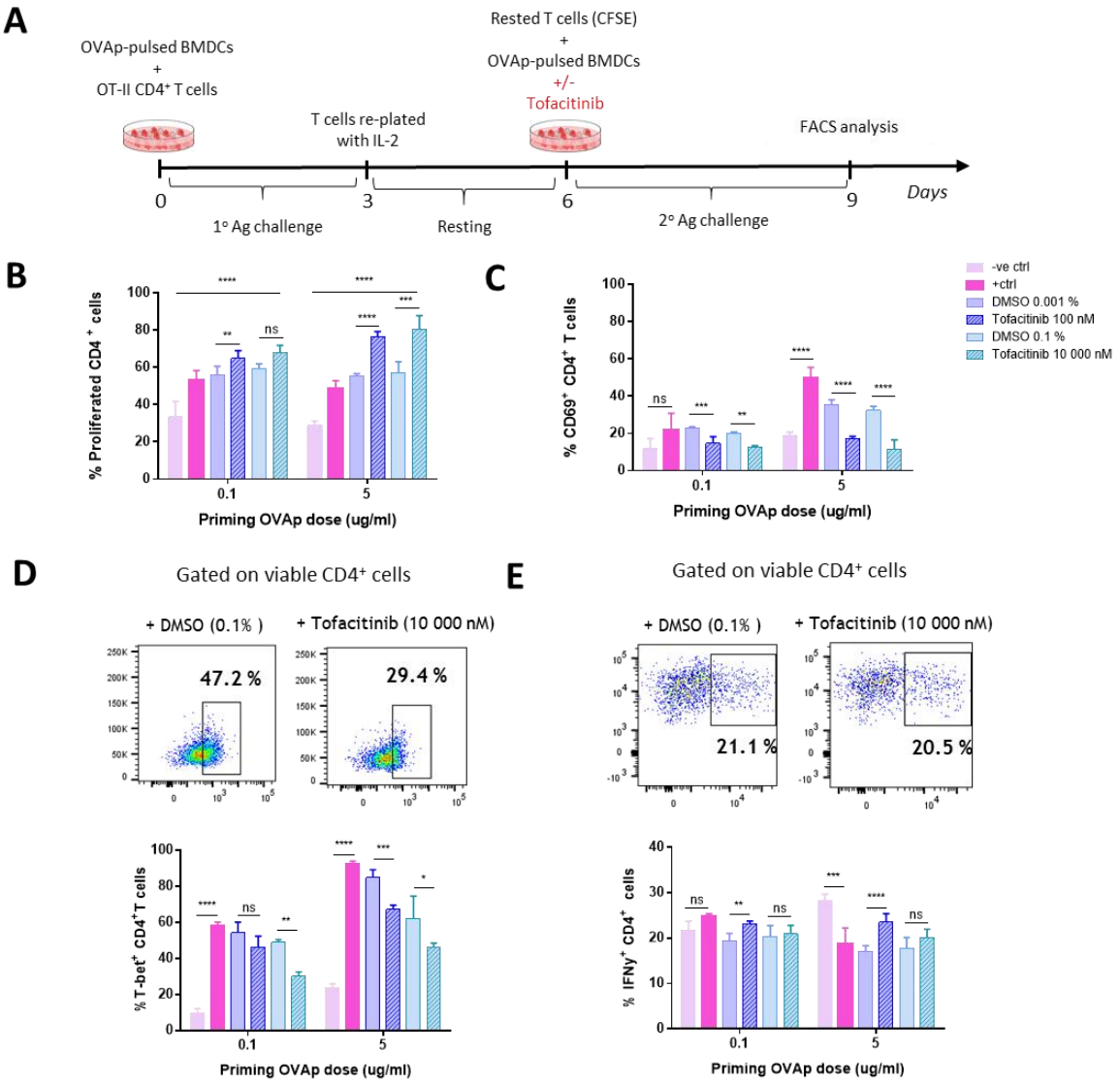


Figure 3-12. Tofacitinib treatment upon reactivation impairs T-bet expression in antigen-experienced CD4⁺ T cell but does not affect their capacity for IFNγ production

CD4⁺ T cells were isolated from lymph nodes and spleens of CD45.1 OT-II mice using STEMCELL EasySep kit and co-cultured with mature DCs in the presence of either 0.1 ug/ml or 5 ug/ml of $OVA^{323-339}$ peptide. After 3 days, cells were washed, re-plated and rested in the presence of IL-2 (1 ng/ml) for another 3 days. Following this, T cells were labelled with CFSE and stimulated with fresh DCs pulsed with the same peptide dose in the presence or absence of tofacitinib. Negative control samples received no antigen during secondary challenge, and positive control samples received antigen with no other treatment. 3 days after secondary challenge, cells were subjected to PMA/Ionomycin treatment in the presence of Brefeldin A and then stained by FACS for intracellular IFNγ production using BD Fixation/Permeabilization kit. Alternatively, cells were directly stained for T-bet expression using Foxp3/Transcription factor staining buffer set. Lymphocytes were gated on viable CD4⁺ T cells and analysed for the percentage of cells which have proliferated (B), CD69 (C) and T-bet expression (D), and IFNγ production (E). Representative scatter plots depict the percentage of T-bet expression (D) and IFNγ producing cells (E) in samples treated with vehicle or 10 000 nM Tofacitinib during the secondary antigenic challenge. Graphs shows mean percentages ± SD of viable CD4⁺ T cells expressing the respective markers. Results are representative of a single experiment for T-bet and a single experiment for IFNγ. Statistical differences between groups were assessed by performing two-way ANOVA and Sidak's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

3.3 Discussion

3.3.1 Tofacitinib interferes with JAK/STAT signalling pathway downstream of both gp130 and γc cytokine receptors

I was able to demonstrate, in both dendritic cell monoculture and DC-$CD4^+$ T cell co-culture, an abrogation of IL-6-induced STAT3 phosphorylation following cell pre-treatment with tofacitinib. Similarly, tofacitinib potently inhibited STAT3 and STAT5 phosphorylation in the $CD4^+$ T lymphocytes stimulated with IL-6 and IL-2 cytokines, respectively. In line with this, an extensive study by Ghoreschi *et al.*[305] also showed tofacitinib was capable of interfering with the signalling of both IL-2 and IL-6 in murine $CD4^+$ T cells by abolishing phosphorylation of respective STAT proteins. Also, the study observed the same effect of the drug on IL-6-dependent pSTAT3 phosphorylation in human PBMC-derived $CD3^+$ T cells. While the purpose of this experiment was to mainly confirm the ability of tofacitinib to inhibit its target pathway in our co-culture system, I have also demonstrated, although indirectly (via STAT activity), the ability of the drug to effectively inhibit not only JAK3 kinase (upstream of STAT5), for which tofacitinib has the highest potency, but also the signalling via JAK1 and JAK2, required for IL-6 signalling via STAT3.

3.3.2 Tofacitinib does not affect naïve $CD4^+$ T cell activation and has a modest effect on their proliferative potential

The expression of activation markers is one of the changes detected in the T cell phenotype shortly after the priming event. In my co-culture, the exposure of naive $CD4^+$ T cells to tofacitinib throughout priming had no impact on the expression of early activation marker CD69 or late activation markers CD44 and CD62L. One of the functions of CD69 is the retention of newly activated T cells in the lymph node[488]. CD62L expression allows naïve CD4 T cells to enter the lymph node and is downregulated upon cell priming to promote activated cell

migration out of the lymph node, while the upregulation of CD44 potentiates the entry of the activated cells from the circulation to the inflamed tissue sites.[492,493,494,490] Thus, maintenance of the above activation marker expression levels independent of tofacitinib exposure suggests that the drug-treated cells undergo efficient activation and presumably, in the *in vivo* setting, are able to migrate to the site of inflammation.

Several studies investigating tofacitinib impact on human PBMCs employed CD25 as an activation marker and found its expression levels to be diminished by the treatment[484, 485] and subsequently restored upon drug withdrawal[484]. However, CD25 functions as an IL-2 receptor α chain (IL-2Ra) on activated T cells thus relaying signals controlling cell proliferation. As mine and other studies have shown, tofacitinib treatment prevents T lymphocytes from responding to IL-2 stimulation, which can ,in turn, result in failure to upregulate CD25 expression as a part of IL-2-driven autocrine negative feedback loop.[509] Thus, impaired CD25 expression upon drug exposure is not a direct result of tofacitinib interference with $CD4^+$ T cell priming (activation) by dendritic cells, but rather a consequence of impaired ability of newly activated T cells to respond to the autocrine/paracrine IL-2 signal.

I next assessed tofacitinib impact on $CD4^+$ T cell proliferation following the priming event. Newly activated $CD4^+$ T cells maintained normal levels of proliferation at the physiological drug concentration of 100 nM, which were slightly but significantly reduced at the higher drug concentration. Similarly, tofacitinib exhibited no effect on naïve murine $CD4^+$ T cell proliferation when used at up to 1000 nM concentration in the study conducted by Ghoreschi *et al*.[305] In contrast, even at 100 nM dose the drug markedly reduced proliferation of lymphocytes derived from PBMCs of healthy patient donors and subjected to PHA stimulation *in vitro*. Interestingly, this effect was completely reversed upon drug removal.[484] A comparable dose-dependent effect on proliferation was observed in $CD4^+$ T cells purified from PMBCs and synovium samples from active RA patients and stimulated *in vitro* in the presence of tofacitinib.[306] While in all of the above studies lymphocytes were exposed to the drug only during *in vitro* culture, Sonomoto and colleagues examined lymphocytes from RA patients receiving tofacitinib treatment as a part of clinical trial[510]. After 12 months of

treatment, $CD4^+$ T cell proliferative potential was notably suppressed and correlated with disease activity improvement. Thus, one of the mechanisms of action of tofacitinib appears to be through the suppression of proliferation in both murine and human T lymphocytes, although murine cells appear to require higher drug doses (in the *in vitro* setting) to achieve a significant effect. The mechanism could involve drug inhibition of IL-2 and IL-7 signalling required for cell proliferation, together with reduced expression of their receptors. [484,485,305]

3.3.3 Tofacitinib treatment impairs $CD4^+$ T cell differentiation and function

Due to the ability of tofacitinib to inhibit cytokine signalling in $CD4^+$ T cells, I sought to assess if the drug could also utilize this strategy to interfere with naïve T cell specification towards T helper subtypes and thereby affect lineage-specific cytokine production. Indeed, tofacitinib treatment during priming resulted in the reduction of Th2-specific IL-5 levels, as well as Th1-associated IFNy and Th17 hallmark cytokine IL-17 production in my co-culture. Similar observations were made in another study using naïve murine T cells, which, when exposed to tofacitinib, failed to efficiently differentiate into either of the above Th subtypes and produce lineage-specific cytokines even under specific polarizing conditions.[305] Maeshima *et al.* reported similar dose-dependent effect of the drug on both IFNy and IL-17 production by $CD4^+$ T cells from synovium and peripheral blood of RA patients.[306] However, the latter study is not directly comparable as the examined T cell population being heterogeneous and containing activated T cells alongside with their naïve counterparts. Thus, my observations confirmed the ability of tofacitinib, due to its broad inhibitory effect on cytokine signal transduction, to non-selectively interfere with IFNy, IL-5, and IL-17 production, and hence presumably the differentiation into respective Th1, Th2 and Th17 subsets. The clinical efficacy of tofacitinib in RA might be, therefore, at least in part explained by the drug limiting generation of functional pathogenic IFNy-producing Th1 and IL-17 producing Th17 cells.

Alongside alterations in Th subset-specific cytokine production, the production of IL-2 was also affected by the drug treatment. The observed increase in IL-2 production was likely a consequence of IL-2 receptor signalling inhibition and the resulting disruption of a negative feedback loop.[499] While IL-2 is important in controlling $CD4^+$ T cell proliferation and survival during priming, it can also act as an additional signal supporting T cell polarization. IL-2 was shown to induce the expression of IL-12 receptor, beta 2 (IL-12Rß2), thus enhancing naïve (Th0) T cell sensitivity for IL-12, which drives terminal Th1 differentiation. Likewise, IL-2 was observed to directly regulate Th2 polarization by promoting both IL-4 and IL-4Rα expression by $CD4^+$ T cells during priming. Th17 differentiation, on the other hand, was suppressed by IL-2, although the cytokine was capable of driving clonal expansion of the Th17 lineage cells once they were fully polarized[511]. In the context of this data, interference of tofacitinib treatment with IL-2 signalling in naïve $CD4^+$ T cells might be viewed as an additional checkpoint for preventing their differentiation into 'pathogenic' T helper cells.

IL-6 plays an important role in RA pathogenesis, and its importance is highlighted by the clinical efficacy of treatments targeting the IL-6 receptor. While in naïve murine T cell monoculture tofacitinib appeared to profoundly inhibit the generation of IL-6[305], I did not observe any changes in IL-6 levels in the co-culture setting. This is likely to result from the presence of dendritic cells in my co-culture, which are also a source of IL-6. Thus, in my current system, it is not possible to establish if tofacitinib impairs IL-6 production by $CD4^+$ T cells.

I also detected IL-10 in our co-culture supernatant, which might be produced by naïve cells polarized towards Treg subtype. This observation of IL-10 production and thus presumably Treg generation preserved during tofacitinib treatment is rather controversial, as the signalling through IL-2-STAT5 axis and the resulting induction of associated transcription factor Foxp3 expression, prerequisite for Treg differentiation, would be inhibited by the drug.[512] While at present the direct effect of tofacitinib on *de novo* Treg differentiation has not been addressed to help contextualise my findings, several studies demonstrated the drug to re-direct dendritic cells towards tolerogenic phenotype. The tolerized dendritic cells, in turn, enhanced $CD4^+Foxp3^+$ Treg differentiation *in vitro*[513], and, when adoptively transferred into an experimental autoimmune

encephalomyelitis (EAE) mouse model, reduced Th1/Th17 cell population size and their functional capacity in favor of Treg expansion.[514] Remarkably, already differentiated $CD4^+$ $Foxp3^+CD25^{bright}$ Treg cells from kidney transplant patients receiving tofacitinib therapy were reduced in numbers, but preserved their suppressive activity and demonstrated lesser sensitivity to treatment than the effector T cells.[506] Based on my preliminary findings and the published data, it could be speculated that in addition to interfering with $CD4^+$ T cell differentiation into T helper subtypes and their subsequent 'pathogenic' function, tofacitinib treatment could provide an additional benefit of potentially unaltered generation and preserved function of existing Treg cells.

I further demonstrated that the particularly notable reduction in IFNγ production was a result of tofacitinib abolishing Th1 polarization through suppressing T-bet expression. This finding comes in line with the requirement of functional IFNγ signalling through activated STAT1 to initiate T-bet expression, which in turn leads to enhanced IFNγ production, as well as induction of IL-12Rβ2 expression. In turn, these result in subsequent T-bet expression enhancement, the establishment of IFNγ self-enhancing loop and cell sensitization to IL-12 stimulation[515]. Inhibition of IFNγ signalling by tofacitinib thereby precludes initial T-bet expression and abolishes the successful establishment of mature Th1 phenotype. Similar changes were detected following naïve murine $CD4^+$ T cell exposure to the drug, which exhibited dose-dependent T-bet reduction comparable with that in $CD4^+$ T lymphocytes from STAT1-deficient mice.[305] Thus, tofacitinib has a capacity to effectively suppress the generation of Th1 helper cells, which, particularly in the context of Th1-driven pathology such as acute graft-versus-host disease (GvHD), is shown to prevent lethal outcome while extending graft survival.[516]

3.3.4 Functional impairment following exposure to tofacitinib during priming persists upon CD4$^+$ T cell re-activation

I subsequently showed that the cells compromised by tofacitinib treatment in their differentiation and associated functional state during priming remain functionally diminished after secondary antigen exposure when the drug is withdrawn. The ability of the untreated cells to exhibit a normal 'memory' response upon re-activation is determined by the presence of open chromatin sites in the enhancer and promoter regions of *Ifng* gene, which could be easily accessed initially by NF-κB and subsequently replaced by T-bet to rapidly re-initiate IFNy production[517,518]. However, since the drug treatment abolishes CD4$^+$ T cell ability to properly undergo priming and thus (likely) prevents the acquisition of permissive chromatin state at both *Tbx21*/*T-bet* and *Ifng* gene promoters, the cells might require more time for the above gene expression *de novo* after drug withdrawal. Also, the magnitude of their response to secondary activation might also be compromised for this reason. However, our results do not clarify if these compromised cells might recover their full function over time, or whether due to chromatin remodelling being compromised during initial antigenic challenge the cells will permanently remain in a functionally immature state. A comparison of epigenetic profiles of control and drug-treated cells would likely provide more insights into their functional potential. Replicating this experiment in the *in vivo* context might also add to the evaluation of the CD4$^+$ T cell pathogenic potential after the treatment termination.

Some attempts have been made to elucidate cell fate upon drug removal using human PMBCs. Piscianz *et al*. observed the recovered responsiveness of lymphocytes to re-activation after tofacitinib withdrawal in the *in vitro* culture, and while some cytokine production remained inhibited, the production of IL-2, IL-13 and TNFα was preferentially restored.[484] Another study demonstrated that IFNy-producing CD4$^+$ T cells collected from healthy individuals 4 weeks after termination of tofacitinib therapy recovered their responses to antigenic stimulation after transient suppression of their function during treatment.[483] Due to both studies utilizing the CD4$^+$ cells of both naïve and activated phenotype and exerting no control over the presence of the drug during cell priming, their

results cannot be directly correlated with my findings. Following these preliminary results, it would be important to further investigate, possibly in the *in vivo* setting, whether tofacitinib treatment permanently impairs $CD4^+$ T cells function when present at priming or whether the drug withdrawal may lead to recovery of their functional capacity. This would help to inform the potential adjustment of the therapeutic regimens for both tofacitinib monotherapy and combined treatment with other immunosuppressive agents.

3.3.5 Antigen-experienced cells preserve their function after tofacitinib exposure

In the co-culture system employed, I primed naive $CD4^+$ T cells and after a brief resting phase re-stimulated them with their cognate antigen, adding tofacitinib at this stage. Upon re-activation, T cells sustained their normal IFNγ production levels despite the notable impairment in their T-bet expression. Since these cells were primed in the absence of the drug, they would be able to normally initiate T-bet expression which is essential for commitment towards Th1 lineage.[507] However, while T-bet activity is required during this commitment phase to drive Th1 lineage-specific gene expression and chromatin remodelling of the *Ifng* locus, the fully mature Th1 cells and their descendants were reported to retain heritable chromatin modification and can re-express IFNγ independently of T-bet[519]. A detailed analysis of temporal T-bet requirement in mature $CD4^+$ T cells by Lai *et al.*[518] has conversely demonstrated that whereas the rapid IFNγ production by previously activated $CD4^+$ T cells was driven by NF-κB transcriptional activity 6 h after re-activation, T-bet was required at later time points to sustain cytokine response. Similarly, Jun Kui Chen (thesis, 2017) has shown IFNγ production by Th1 polarized cells following T-bet ablation to be initially preserved and to decline in the long-term in the absence of T-bet. Since in my experiment re-activated T cells retained relatively high levels of T-bet expression, their IFNγ production might remain partly dependent on T-bet activity and possibly partly compensated by the alternative mechanism such as NF-kB pathway, potentially unaffected by tofacitinib treatment.

Interestingly, the production of IFNγ in antigen-experienced $CD4^+$ T cells following re-activation in the presence of the drug was preserved. This potentially might be explained by either lesser susceptibility of mature effector $CD4^+$ T cells to the cytokine signalling inhibition by the drug, or lower threshold required for their activation that for differentiation of the naïve cells. Another possible reason might be that the recruitment of the alternative signalling pathways independent of JAK kinase activity might be sufficient for effectively inducing IFNγ production. However, the existing studies with human PBMCs seem to contradict my findings. A study by Sewgobind *et al*. demonstrates tofacitinib to potently inhibit IL-2 induced STAT5 phosphorylation in effector T cells and suppress their function (proliferative ability).[506] Moreover, $CD4^+$ lymphocytes isolated from peripheral blood and synovium of patients with active RA exhibited a reduced capacity for IFNγ and IL-17 production upon CD3/CD28 stimulation in presence of tofacitinib *in vitro*.[306] Similar results were obtained with $CD4^+$ cells from healthy donors receiving short-term tofacitinib treatment.[483] The analysis of their PBMCs showed a profound reduction in all subsets of activated T cells, including $CD4^+$ T cell compartment. Moreover, exposure to tofacitinib resulted in a short-term decrease in IFNγ production by IFNγ-producing activated cells upon both antigen-specific and CD3/CD28 re-stimulation. While the above data cannot be directly correlated with my findings as they mostly employ heterogeneous T cell populations, overall, they might suggest the ability of tofacitinib to impact effector T lymphocyte functional ability. Replicating the above experiment in the *in vivo* setting might provide more reliable results.

Since one of my main findings in this chapter was the ability of tofacitinib to interfere with $CD4^+$ T cell differentiation towards Th1 subset, I have proceeded to enquire if the same effect could be observed in the inflammatory setting *in vivo*.

4 Investigating the impact of tofacitinib administration on $CD4^+$ T cell-DC cross- talk *in vivo*

All animal experiments were done with help from Bob Benson.

4.1 Introduction

In the previous chapter, I demonstrated that CD4$^+$ T cells exposed to tofacitinib during priming with their cognate antigen *in vitro,* failed to effectively differentiate and exhibited a profoundly diminished capacity for IFNy production. However, the *in vitro* setting represents a highly controlled environment where the drug is easily accessible to the target cells. *In vivo,* the action of the compound will be influenced by its pharmacokinetic and pharmacodynamic properties, as well as biodistribution and bioavailability in relation to the cells of interest and target tissue. Thus, my next step was to determine whether the inhibitory effect of tofacitinib on CD4$^+$ T cell differentiation and function could be translated *in vivo*.

Multiple studies have employed various rodent models of arthritis to assess tofacitinib efficacy *in vivo*. These mostly investigated the broader therapeutic effect of tofacitinib in the context of systemic inflammatory mediator levels, histological and structural changes in the joint, and clinical disease score[305,310,311]. Apart from a study in the SKG arthritis model, where the reduction in disease severity following tofacitinib treatment was associated with reduced levels of IFNy and IL-17, and thus potentially impaired Th1 and Th17 cell function[312], there has been little specific focus on the activity of CD4$^+$ T cells *in vivo*. The relevant human studies investigating tofacitinib impact on CD4$^+$ T cell function, discussed in the previous chapter, employed PBMCs containing CD4$^+$ T cells at different activation stages and were thereby unable to dissect the effect of the drug specifically on naïve CD4$^+$ T cells.[483,484,485] Thus, to our knowledge, there are no studies to date that directly address the impact of tofacitinib on naïve CD4$^+$ T cells *in vivo*.

To this end, I sought to replicate the *in vitro* TCR-transgenic OT-II CD4$^+$ T cell activation studies (Chapter 3) in the *in vivo* setting by means of adoptive transfer experiments. As the majority of these T cells express a TCR specific for chicken OVA$^{323-339}$ peptide in the context of I-Ab, they can be challenged *in vivo* in a controlled manner and assessed in the context of antigen-specific response. Adoptive transfer circumvents issues relating to detection of low frequency of

endogenous antigen-specific cells, enabling measurement of antigen-specific T cells responses such as clonal expansion and cytokine production *in vivo*, and assessment of any changes arising through drug treatment. Use of the congenic marker CD45.1 on OT-II $CD4^+$ T cells allowed them to be distinguished from the endogenous T cells, which express CD45.2 isoform in the (C57BL/6) recipient mice. Together with all the above, the naïve state of these OT-II $CD4^+$ T cells makes this transgenic system a desirable tool for addressing the questions of this chapter.

The key aims of this chapter are the following:

- To determine the capacity of Tofacitinib to inhibit the JAK/STAT pathway in $CD4^+$ T cells *in vivo*
- To investigate the impact of Tofacitinib treatment on the outcome of antigen-specific priming of $CD4^+$ T cell *in vivo*
- To assess whether tofacitinib treatment influences endogenous $CD4^+$ T cell functional capacity *in vivo*

4.2 Results

4.2.1 Tofacitinib citrate supresses STAT phosphorylation in CD4$^+$ T cells upon cytokine stimulation with comparable efficacy to tofacitinib base form *in vitro*

For conducting the *in vivo* experiments, I employed an alternative formulation of the drug, tofacitinib citrate salt. The manufacture of the drug in the crystalline citrate salt form enhances its dissolution rate and solubility in the intestine, compared to a poorly soluble base form, while preserving the pharmacological properties of the compound[520], and thus a citrate salt form of tofacitinib was selected for *in vivo* testing and clinical use. To confirm that the molecular mode of action of this drug formulation was comparable to that of the tofacitinib base, I have first assessed its capacity to inhibit STAT protein phosphorylation *in vitro* prior to employing tofacitinib citrate form in the *in vivo* setting. For this purpose, mature dendritic cells were co-cultured with naïve OT-II CD4$^+$ T cells overnight in the presence of OVA$^{323-339}$ peptide and with either a vehicle or 10 000 nM tofacitinib citrate. 24 hours later, cells were stimulated with either IL-6 or IL-2 for 15 min and subsequently assessed by Phospho-flow cytometry for levels of phosphorylated STAT3 or STAT5, respectively. CD4$^+$ T cells stimulated with their cognate antigen in the presence of a vehicle and respective cytokine demonstrated a subtle increase in pSTAT3 levels and a more pronounced increase in pSTAT5 levels relative to those in the co-culture in the absence of the peptide (Figure 4-1). The addition of tofacitinib citrate inhibited CD4$^+$ T cell responsiveness to either cytokines produced by cells or those added to the culture, resulting in pSTAT3 level reduction below the baseline alongside a notable decrease in pSTAT5 levels. Unlike lymphocytes treated with tofacitinib base, the CD4$^+$ T cell population exposed to the citrate drug form did not segregate into distinctive pSTAT5-positive and pSTAT5-negative fractions but rather presented as a more uniform population. This intra-experimental variability could be due to differential CD4$^+$ T cell response to the cytokine stimulation or the inhibition by distinct tofacitinib formulations. Overall, both tofacitinib citrate and base (see Fig.3-3) blocked cytokine signalling and downstream STAT protein phosphorylation in CD4$^+$ T cells with similar efficacy.

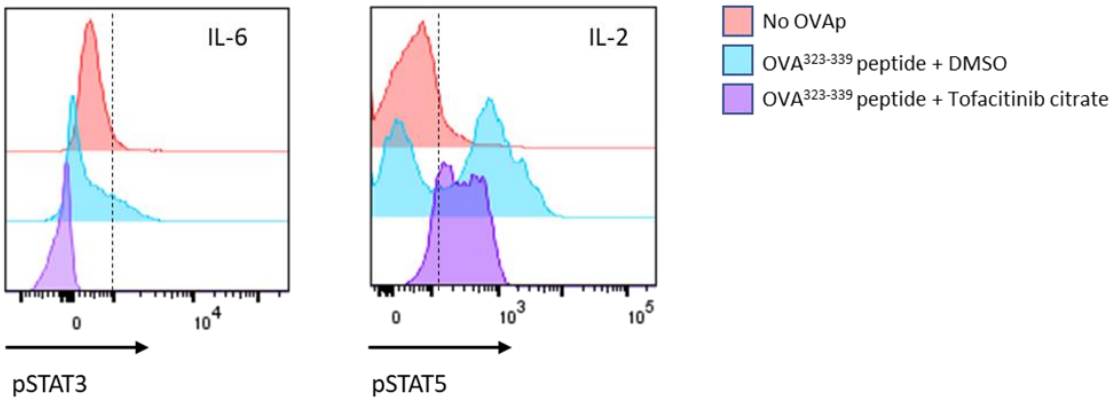


Figure 4-1. Tofacitinib citrate effectively supresses STAT3 and STAT5 phosphorylation upon cytokine stimulation of CD4⁺ T cells *in vitro.*

Matured bone marrow-derived DCs were incubated with or without 5 ug/ml of $OVA^{323-339}$ peptide for 2 h at 37°C. $CD4^+$ T cells were isolated from OT-II mice lymph nodes and spleens using STEMCELL EasySep kit and cultured with DCs overnight in the presence or absence either 10 000 nM tofacitinib citrate dissolved in DMSO vehicle, vehicle, or no treatment. Cells were subsequently stimulated with either IL-2 or IL-6 (both at 100 ng/ml) for 15 min at 37C, immediately fixed and stained intracellularly for phosphorylated STAT3 and STAT5 for flow cytometric analysis. Histograms represent levels of pSTAT3 and pSTAT5 in $CD4^+$ T cell populations in response to IL-6 and IL-2 stimulation, respectively. Negative control (pink, no OVAp) received no external stimuli and depicts basal levels of both phospho-proteins in non-activated $CD4^+$ T cells. Dashed lines represent approximate threshold for STAT phosphorylation from the baseline. The histograms are representative of the phosphorylated STAT levels of the samples from a single experiment.

4.2.2 Tofacitinib citrate exposure during priming has an impact on CD4$^+$ T cell viability, activation, and function comparable to tofacitinib base form *in vitro*

After demonstrating tofacitinib citrate capacity to block cytokine signalling in CD4$^+$ T cells, I sought to establish if it also exerted the same effect as tofacitinib base on CD4$^+$ T cell phenotype and function, when present during priming. Naive CD4$^+$ T cells were co-cultured with OVA$^{323-339}$-pulsed dendritic cells in the presence of increasing concentrations of vehicle or tofacitinib citrate. For direct comparison, some samples were treated with a 1000 nm dose of tofacitinib base, previously shown to have an impact on the parameters of interest. After 3 days of co-culture, CD4$^+$ T cells were assessed by flow cytometry for their viability, activation status and capacity for IFNy and IL-2 production.

Across all the treatment doses in both vehicle and drug groups, CD4$^+$ T cell viability appeared to be relatively low compared to the unstimulated control samples (Figure 4-2A). Since all of the peptide-treated samples have a rather low percentage of viable cells regardless of treatment, it could potentially be a consequence, of the antigen-specific interaction, rather than the type of treatment. At 100 nM and 1000 nM tofacitinib citrate doses the percentage of viable cells increased significantly, and this change is comparable in tofacitinib base-treated samples, contrary to what was observed in the previous co-cultures. However, at a maximal dose of 10 000 nM tofacitinib citrate leads to a profound reduction in cell viability, consistent with the previously observed effect of the treatment with base drug formulation. When assessing CD4$^+$ T cell activation, CD44 expression was slightly but significantly upregulated in the tofacitinib citrate-treated cells compared to vehicle controls at both 1000 nM and 10 000 nM doses, with a similar effect observed in samples receiving tofacitinib base (Figure 4-2B). This effect was observed previously but should be interpreted with caution regarding its biological relevance.

Most importantly, the impact of tofacitinib citrate and base forms on the CD4$^+$ T cell capacity for cytokine production was closely comparable. Tofacitinib citrate treatment induced increased IL-2 production at 100 nM dose and this effect

persisted at higher drug doses, replicating the effect of the tofacitinib base (Figure 4-2C). Similarly, tofacitinib citrate demonstrated a dramatic inhibitory effect on IFNy production by $CD4^+$ T cells starting at the 100 nM dose, with the extent of inhibition comparable to that of the base formulation (Figure 4-2D). Collectively, this data demonstrates that the changes in $CD4^+$ T cell phenotype and function, resulting from tofacitinib citrate presence during their priming, are closely comparable to those induced by the tofacitinib base.

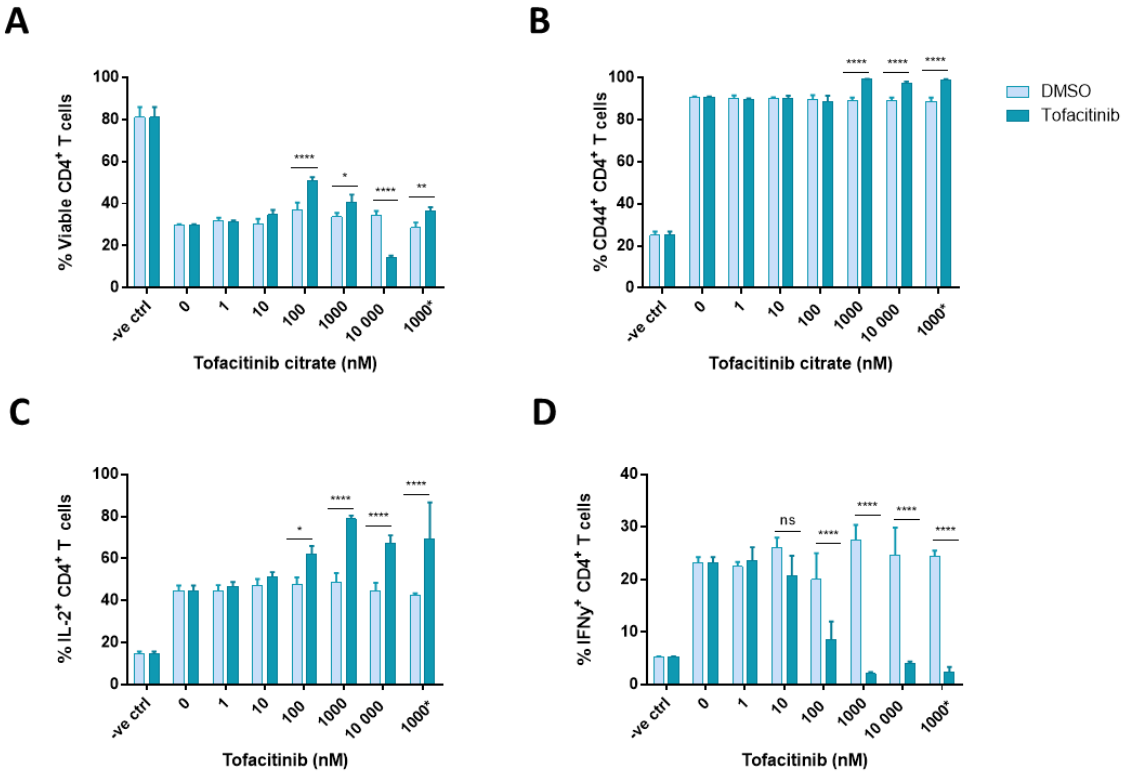


Figure 4-2. Tofacitinib citrate treatment during priming has similar effect to tofacitinib base form on CD4⁺ T cell viability, activation, and cytokine production capacity *in vitro*.

Matured bone marrow-derived DCs were incubated with 5 ug/ml of $OVA^{323-339}$ peptide for 2 h at 37ºC. $CD4^+$ T cells were isolated from OT-II mice lymph nodes and spleens using STEMCELL EasySep kit and cultured with DCs in the presence or absence of either increasing concentrations of tofacitinib citrate, vehicle, or no treatment. Positive control samples were treated with 1000 nM of tofacitinib base (designated 1000*). Negative control samples received no peptide and were left untreated. After 72 h incubation, cells were stimulated with PMA (10 ng/ml) Ionomycin (500 ng/ml) in the presence of Brefeldin A for 4 h at 37ºC 5% CO_2. Thereafter, cells were stained by FACS for viability, CD4 and CD44 marker expression, followed by fixation/permeabilization step and fluorescent labelling of intracellular IFNy and IL-2 cytokines. Lymphocytes were gated on viable $CD4^+$ T cells expressing either, CD44, IL-2 or IFNy. Graphs show mean percentages ± SD of viable $CD4^+$ T cells (A), $CD4^+$ T cells positive for CD44 (B), and $CD4^+$ lymphocytes expressing either IL-2 (C) or IFNy (D). Results are representative of a single experiment. Statistical differences between groups were assessed by performing two-way ANOVA and Sidak's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05, ** ≤ 0.01, **** ≤ 0.0001. ns = not significant.

4.2.3 Single tofacitinib citrate dose administration *in vivo* reduces STAT3 phosphorylation levels in whole blood leukocytes at both basal state and following *ex vivo* cytokine stimulation

Upon demonstrating the efficiency of tofacitinib citrate treatment *in vitro* and its comparable effects to tofacitinib base formulation, it was next required to test the drug efficacy *in vivo*. Prior to employing a relatively intricate 3-day adoptive transfer model, I sought to first demonstrate that the administration of tofacitinib *in vivo* can effectively inhibit the signalling through the JAK/STAT pathway. According to the pre-clinical pharmacokinetic/pharmacodynamic (PK/PD) tofacitinib profiling, a single oral dose of 50 mg/kg tofacitinib citrate sufficiently inhibits signalling mediated by JAK1-containing heterodimers (JAK1/JAK3 and JAK1/JAK2), and nearly complete inhibition of signalling is achieved 1 h post-dose.[344] Based on the publication by Ghoreschi *et al*.[305] and personal communication with the authors, I decided to use 50 mg/kg/day of tofacitinib citrate to be administered across two doses. Accordingly, a single dose equivalent of 25 mg/kg was employed in this preliminary experiment.

C57BL/6 mice were administered with either a single dose of DMSO or tofacitinib citrate (25 mg/kg) dissolved in 0.5% methylcellulose/0.025% Tween20 vehicle solution and were sacrificed approximately 40 minutes later. For each mouse, the whole blood was collected by cardiac puncture and either received a surface antibody cocktail alone or was also simultaneously stimulated *ex vivo* with IL-6. After 15-minute incubation at 37^{o}C, cells were immediately fixed and stained for phosphorylated STAT3 protein. Flow cytometry analysis revealed that the *in vivo* exposure to tofacitinib citrate did not notably affect the background pSTAT3 levels in circulating $CD4^{+}$ T lymphocytes, but has profoundly reduced the percentage of pSTAT3 protein in $CD4^{-}$ leukocytes in cytokine-unstimulated samples (6.13 %), compared to vehicle-treated controls (28.4 %) (Figure 4-3A). The proportion of pSTAT3-positive $CD4^{+}$ T cells increased by 5.43 % from the background levels with IL-6 stimulation in the presence of a vehicle, in contrast to less than 1% in drug-treated cells. Similarly, drug exposure reduced the percentage of $CD4^{-}$ pSTAT3-expressing cells upon cytokine stimulation from

21.4% to 15.47%. The effect of tofacitinib treatment is also well illustrated in the histograms contrasting the levels of pSTAT3 in unstimulated and stimulated whole blood cells (Figure 4-3B). The difference between the fluorescence intensity of the peaks, representative of unstimulated and stimulated cells, demonstrates the diminished capacity of both $CD4^+$ and $CD4^-$ cells to respond to IL-6 signals and to subsequently induce effective STAT3 phosphorylation following the drug exposure.

Collectively, the above results show that a single oral dose of 25 mg/kg tofacitinib citrate resulted in a detectable reduction of the background pSTAT3 levels *in vivo* in the unstimulated whole blood cells. Moreover, when the cells exposed to the drug were stimulated with IL-6 *ex vivo*, they retained their reduced responsiveness to cytokines manifested by the impaired ability for STAT3 phosphorylation.

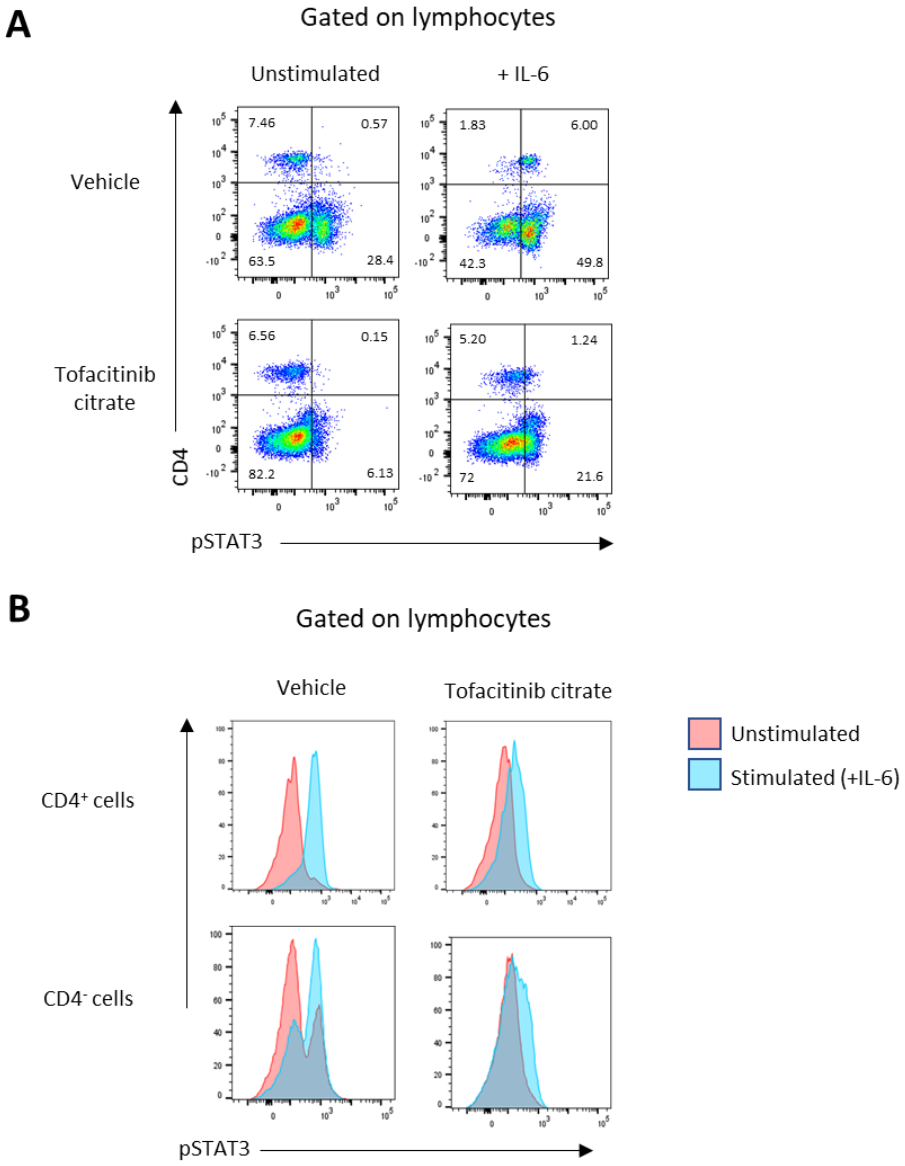


Figure 4-3. A single tofacitinib citrate dose administration *in vivo* reduces STAT3 phosphorylation in the whole blood cells at both basal state and following *ex vivo* cytokine stimulation.

C57BL/6 mice were administered with a single dose of either vehicle (5% DMSO in methylcellulose solution) or tofacitinib citrate (in the same solution at 25 mg/kg) by oral gavage and sacrificed 40 minutes later. Whole blood was obtained by cardiac puncture, split into two parts for each treatment group and incubated with surface antibody cocktail either without or with IL-6 (100 ng/ml) for 15 min at 37ºC 5% CO_2. Cells were then immediately fixed, permeabilized and stained intracellularly for phosphorylated STAT3 for flow cytometric analysis. Lymphocytes were gated based on FSC and SSC and analysed for their expression of CD4 and pSTAT3. Representative scatter plots show the levels of pSTAT3 in both $CD4^+$ and $CD4^-$ cell populations present in the whole blood from vehicle and drug-treated mice either at their basal state or after IL-6 stimulation (A). The numbers indicate percentage of cells in each quadrant, with percentage of $CD4^+$ and $CD4^-$ cells expressing pSTAT3 shown in the top right and bottom right quadrants, respectively. Representative histograms demonstrate the shift in pSTAT3 levels upon *ex vivo* cytokine stimulation individually in $CD4^+$ and $CD4^-$ cell populations from vehicle and drug-treated animals (B). The experiment was done once with a single animal for each vehicle and drug treatment groups.

4.2.4 Tofacitinib citrate effectively diminishes background STAT3 phosphorylation in the whole blood leukocytes but does not affect proliferation or activation of OT-II $CD4^+$ T cells in the adoptive transfer model.

The next step was to perform the adoptive transfer experiment. While this experiment aimed to establish the impact of the tofacitinib citrate treatment on differentiation and function of antigen- specific $CD4^+$ T cells, it also provided groundwork for the more technically challenging and biologically complex RA mouse model experiment (Chapter 5). Naïve $OVA^{323-229}$-specific $CD4^+$ T cells isolated from OT-II transgenic mice were transferred into C57BL/6 recipients and received the first 25 mg/kg dose of vehicle or tofacitinib citrate. The aim of this was to saturate the system with drug prior to *in vivo* priming of the transferred $CD4^+$ T cells. The following day recipient mice were challenged in the footpad with either a mixture of LPS and $OVA^{323-229}$ peptide or LPS alone. Several hours after the injection, the challenged feet appeared red and swollen, confirming successful induction of local inflammation. Utilizing $OVA^{323-339}$ instead of the whole protein allowed to circumvent the potential impact tofacitinib treatment might have on the antigen uptake and processing.

While most animal studies employing tofacitinib used osmotic mini pumps, the surgical procedure required for their subcutaneous implantation was regarded to be unnecessarily invasive for conducting this preliminary short-term experiment, and thus oral gavage was chosen as a more optimal method for drug delivery. The vehicle or tofacitinib citrate (at 25 mg/kg/dose) was administered by oral gavage twice daily, with such regimen previously reported to achieve therapeutic efficacy in the CIA mouse model.[344] On day 3 post-challenge, mice received a final single dose of either drug or vehicle followed by sacrifice 40 minutes later.

To confirm the inhibitory effect of tofacitinib regimen on the JAK/STAT pathway signalling, whole blood was collected from mice by cardiac puncture and stained for surface markers, followed by intracellular staining for pSTAT3. Flow cytometric analysis established the percentages of $CD4^+$ and $CD4^-$ circulating cells expressing pSTAT3 at their basal state in the vehicle-only treated mice,

which were 2.5% and 23.4%, respectively (Figure 4-4A). In the $OVA^{323-339}$-challenged animals receiving vehicle, the fraction of pSTAT3-positive cells in both cell populations has notably increased to 8.58% for $CD4^+$ cells and 65 % for their counterparts, correlating with the ongoing antigen-specific inflammatory response. Interestingly, mice receiving a peptide challenge together with tofacitinib treatment exhibited a profound reduction in the percentage of pSTAT3-expressing cells in both populations to the levels below those in the vehicle-only group. Similarly, when depicting changes in pSTAT3 levels individually in $CD4^+$ and $CD4^-$ populations as histograms, peptide treatment in the presence of vehicle revealed notable STAT3 phosphorylation in both cell populations, while tofacitinib treatment retained the pSTAT3 levels at or below the baseline (Figure 4-4B). While I was particularly interested in the effect of the drug on OT-II $CD4^+$ T cell capacity for STAT3 phosphorylation, I could not assess this population individually due to the majority of adoptively transferred $CD4^+$ T cells presumably accumulating in the draining lymph node at this timepoint and thus being represented by a small fraction in the whole blood.

Thereby, I have confirmed that the tofacitinib dose and delivery route chosen for the adoptive transfer experiment effectively inhibited JAK activity leading to STAT3 phosphorylation in the circulating leukocytes within 1 h of drug administration. Together with the data from pharmacokinetic tofacitinib profiling and my *in vitro* studies, these results suggested that the chosen therapeutic regimen would ultimately promote changes in $CD4^+$ T cell responsiveness to cytokine signalling and the associated capacity for effective priming.

Next, I sought to determine whether the effective JAK/STAT pathway inhibition by tofacitinib translated into an impact on the antigen-specific $CD4^+$ T cell ability to undergo activation and clonal expansion. The adoptive OT-II $CD4^+$ T cell transfer and the footpad challenge were performed as described earlier in this section, and tofacitinib citrate (25 mg/kg) was administered once on the day of the adoptive transfer and then twice daily for the following 3 days (Figure 4-5A). On the last day of experiment, mice received a single drug dose and were sacrificed 40 minutes later. This was an optimal experiment termination time point as the peak T cell accumulation in the draining lymph nodes is known to

occur 3(-4) days after the antigenic challenge. Popliteal lymph nodes, draining the foot, were harvested, mashed into a single cell suspension and the $CD4^+$ lymphocytes were first assessed for their proliferation and activation status by flow cytometry. Distinguishable populations of transferred OT-II ($CD45.1^+$) and endogenous ($CD45.1^-$) $CD4^+$ T cells were observed in all three treatment groups, with OT-II cells undergoing notable clonal expansion in response to cognate peptide challenge, which was unaffected by the drug treatment (Figure 4-5B). As expected, transferred OT-II $CD4^+$ T cells also displayed an activated phenotype, with nearly 100% of cells being $CD44^{hi}$ in both $OVA^{323-339}$-challenged animal groups (Figure 4-5C). These observations confirmed that tofacitinib treatment does not affect $CD4^+$ T cell proliferation or activation during priming *in vivo*, reiterating the results obtained *in vitro*.

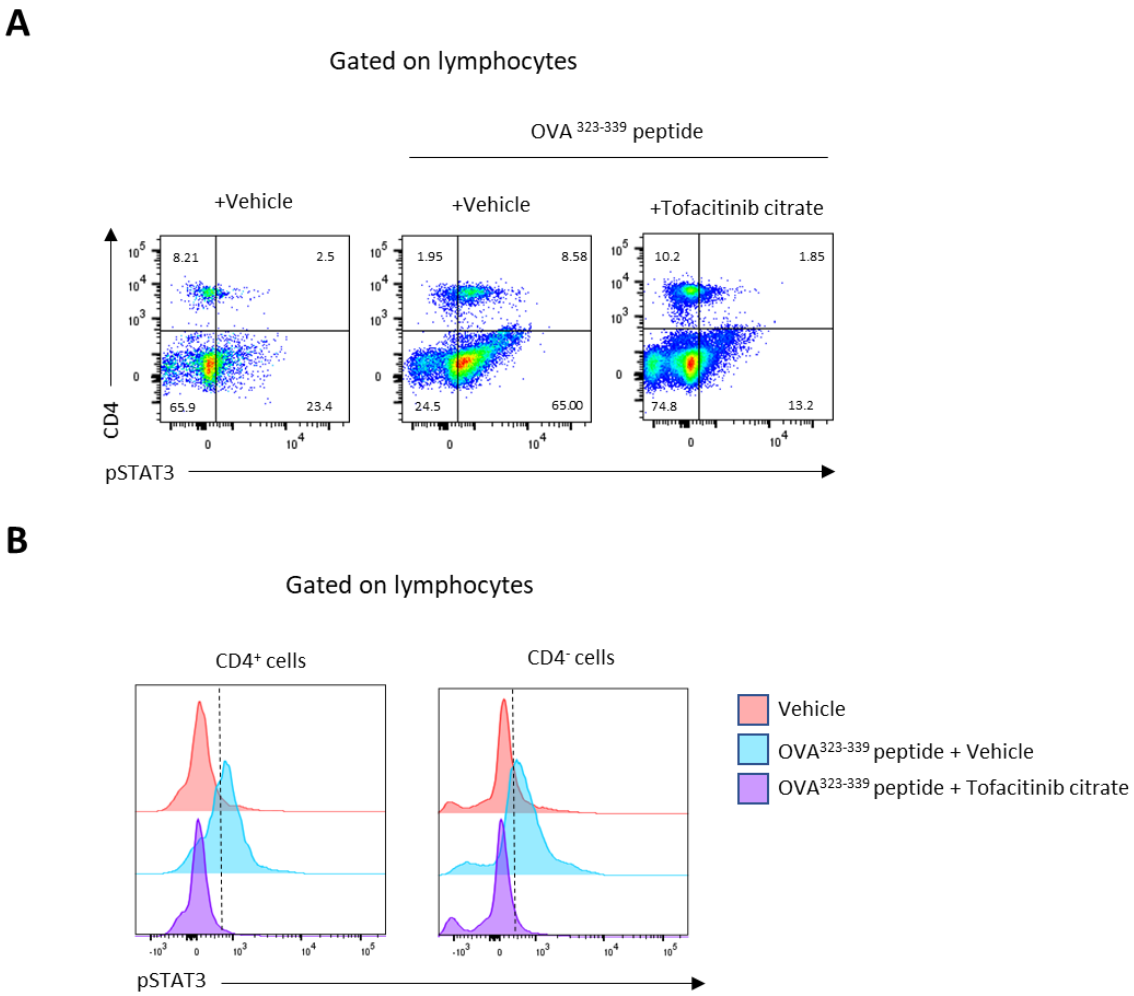


Figure 4-4. Tofacitinib citrate treatment diminishes basal levels of phosphorylated STAT3 in the whole blood cells of adoptive transfer mouse model.

$1x10^6$ of OVA $^{323-339}$-specific $CD4^+$ T cells from transgenic OT-II mice were adoptively transferred into C57BL/6 recipients, which have simultaneously received a single dose of either a vehicle or tofacitinib citrate (25 mg/kg) by oral gavage. After 24 h mice were challenged with 8 ug of LPS and 25 ug of $OVA^{323-339}$ peptide or LPS alone, injected into the footpad. On the day of the challenge and for the next two days mice were administered with either vehicle or tofacitinib citrate (25 mg/kg) twice daily by oral gavage. Three days after the footpad challenge, mice received a single dose of either vehicle or tofacitinib citrate (25 mg/kg) and were sacrificed 40 minutes later. Whole blood was obtained by cardiac puncture and incubated with a surface antibody cocktail for 15 min at 37°C 5% CO_2. Cells were then immediately fixed, permeabilized and stained intracellularly for phosphorylated STAT3. Lymphocytes were gated based on FSC and SSC and analysed for their expression of CD4 and pSTAT3. (A) Flow cytometric analysis of whole blood cells, with scatter plots representing pSTAT3 levels in the whole blood from mice from each treatment group. The numbers indicate percentage on cells in each quadrant, with percentage of $CD4^+$ and $CD4^-$ cells expressing pSTAT3 shown in the top right and bottom right quadrants, respectively. (B) Histograms show levels of pSTAT3 from scatter plots in (A) individually for $CD4^+$ and $CD4^-$ cells. Results represent a single experiment with 3 mice in 'Vehicle only' group and 4 mice in the other two groups. Dashed lines represent the approximate threshold for STAT phosphorylation from the baseline.

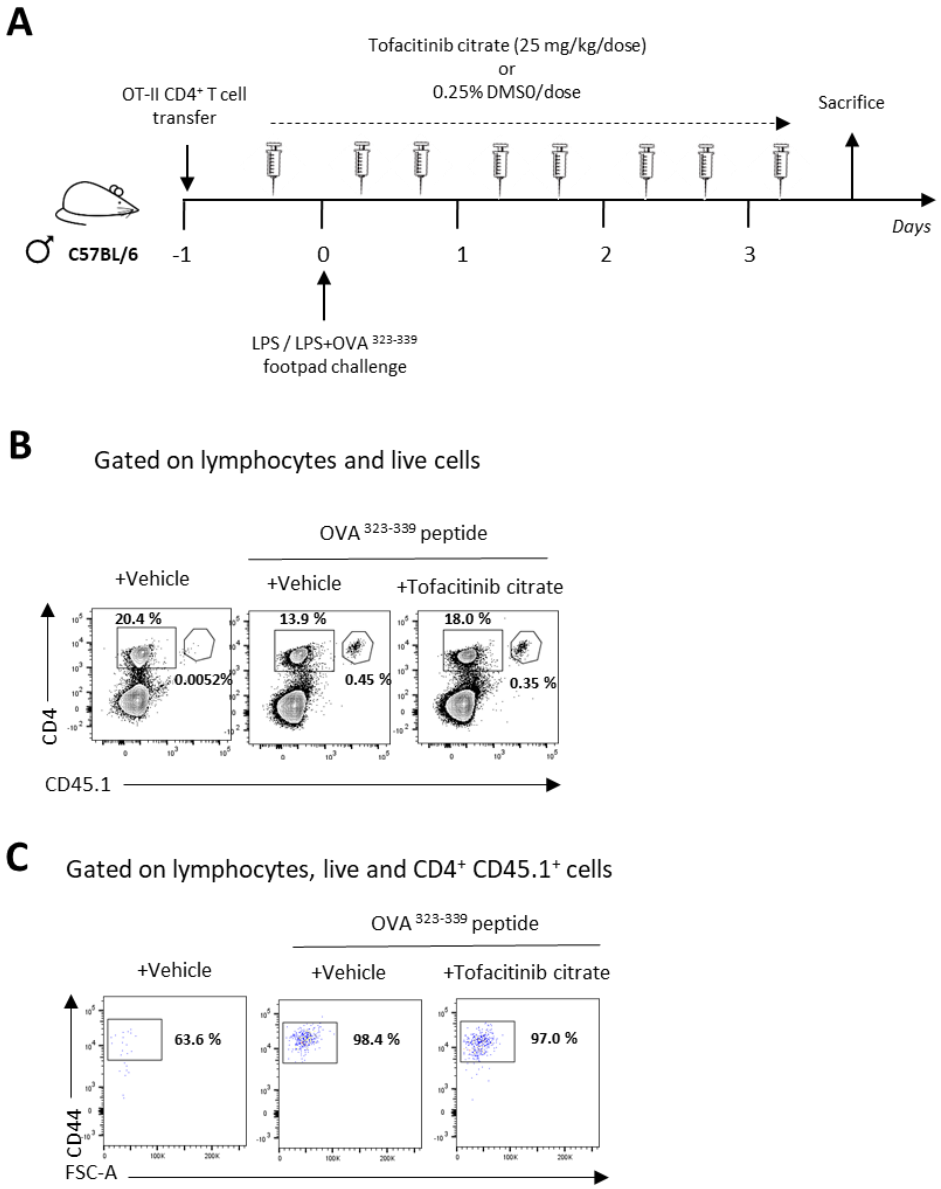


Figure 4-5. Tofacitinib citrate treatment does not affect proliferation or activation of adoptively transferred OT-II CD4⁺ T cells *in vivo.*

C57BL/6 mice received 1x10^6 OT-II CD4$^+$ T cells and 24 h later were challenged with LPS or LPS with OVA$^{323-339}$ peptide in the footpad. Mice received treatment by gavage of either vehicle or tofacitinib citrate (25 mg/kg) once on the day of cell transfer and then twice daily on the consecutive 2 days. On day 3 post-challenge, mice received a single treatment dose and were sacrificed 40 min later (A). Popliteal lymph nodes were removed and mashed into a single cell suspension, with half of the cells from each lymph node stained by FACS for CD4, CD44, and CD45.1 surface markers. Lymphocytes were gated on live cells and analysed for proportions (designated as percentages) of CD4$^+$CD45.1$^+$ OT-II and CD4$^+$CD45.1$^-$ endogenous cells in each treatment group by flow cytometry (B). Adoptively transferred CD4$^+$CD45.1$^+$ cells were then assessed for CD44 expression (B). Results represent a single experiment with 3 mice in 'Vehicle only' group and 4 mice in the other two groups.

4.2.5 Tofacitinib treatment supresses Th1 differentiation in adoptively transferred OT-II CD4$^+$ T cells *in vivo*

Upon demonstrating the absence of tofacitinib impact on OT-II CD4$^+$ T cell ability for clonal expansion and activation, I have next sought to assess whether the adoptively transferred cells could similarly undergo effective priming and differentiation. Flow cytometric intracellular staining of popliteal lymph nodes for T-bet showed between 50% and 80% of the OT-II cells to exhibit transcription factor expression when primed with their cognate peptide in the presence of vehicle, indicating polarization towards Th1 phenotype (Figure 4-6A,B). In the peptide-treated animals receiving tofacitinib treatment, however, there was a significantly lower percentage of T-bet-positive OT-II cells. (Figure 4-6A,B). The mean numbers of T-bet expressing OT-II CD4$^+$ T cells followed the same trend as the mean cell percentages but were not statistically significant (Figure 4-6C).

To assess if the effect of tofacitinib on the differentiation of OT-II CD4$^+$ T cells has compromised their function, cells from popliteal lymph nodes were re-stimulated *ex vivo* and stained for appropriate surface markers, followed by the intracellular IFNy and IL-2 cytokine staining for the assessment by flow cytometry. As expected, upon antigenic challenge a significantly higher percentage of primed OT-II CD4$^+$ T cells exhibited IFNy production, compared to the OT-II cells in the LPS-only challenge group (Figure 4-7A,B). This correlated with the observation of the notable proportion of transferred CD4$^+$ T cells differentiating into Th1 subset following cognate peptide challenge. However, despite the tofacitinib administration notably supressing T-bet expression, the associated reduction in IFNy-producing OT-II cells was not significant (in either percentage or cell number) (Figure 4-7A,B). OT-II cells also markedly upregulated IL-2 production in the OVA peptide-challenged mice treated with vehicle, while the cells from drug-treated mice exhibited further enhancement in IL-2 production (Figure 4-7D,E). This is consistent with the tofacitinib blocking IL-2 signalling and thereby interfering with the STAT5-dependent IL-2 autocrine and/or paracrine negative feedback loop which limits IL-2 production.[499] Nevertheless, the enhanced capacity for IL-2 production by OT-II cells primed in

the presence of the drug was not statistically significant (in either percentage or cell number)(Figure 4-7D,E).

Thereby, tofacitinib administration during priming of adoptively transferred OT-II $CD4^+$ T cells *in vivo* markedly suppressed their T-bet expression and subsequent differentiation towards Th1 subset. However, the capacity of these $CD4^+$ T cells to produce Th1-specific pro-inflammatory cytokine IFNy was not significantly altered. Similarly, the extent of an increase in IL-2 production in the presence of the drug did not reach statistical significance.

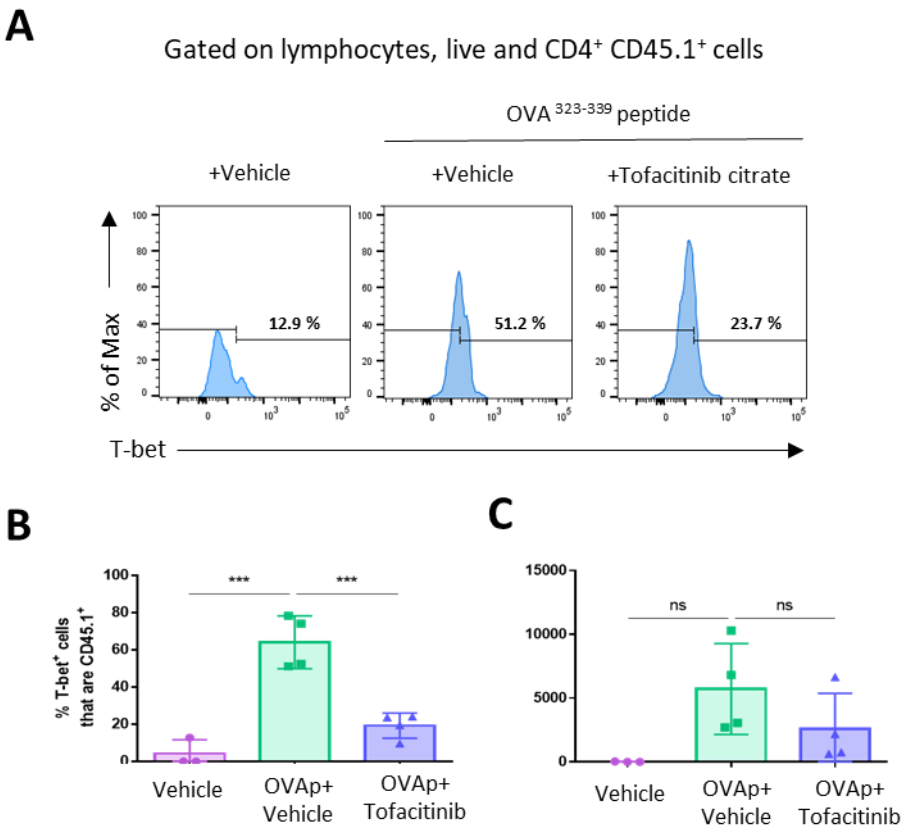


Figure 4-6. Tofacitinib citrate treatment supresses T-bet expression in adoptively transferred OT-II CD4⁺ T cells *in vivo.*

C57BL/6 mice received 1x10^6 OT-II CD4$^+$ T cells and 24 h later were challenged with LPS or LPS with OVA$^{323-339}$ peptide in the footpad. Mice received treatment by gavage of either vehicle or tofacitinib citrate (25 mg/kg) once on the day of cell transfer and then twice daily on the consecutive 2 days. On day 3 after challenge, mice received a single treatment dose, were sacrificed 40 min later and their popliteal lymph nodes were removed and mashed into a single cell suspension. Half of the cells from each lymph node were stained by FACS for CD4, CD44 and CD45.1 surface markers, followed by intracellular staining for transcription factor T-bet. Lymphocytes were gated on live cells and CD4$^+$CD45.1$^+$ cells were identified as the adoptively transferred OT-II lymphocytes. Histograms are representative of the percentage of T-bet positive cells in the OT-II cell population of each treatment group (A). Graphs depict mean ± SD of percentage (B) and number (C) of T-bet-positive CD4$^+$CD45.1$^+$ cells. Results represent a single experiment with 3 mice in 'Vehicle only' group and 4 mice in other the two groups. Statistical differences between groups were assessed by One-way ANOVA and Turkey's multiple comparison test in GraphPad prism. ***designates a *p*-value of ≤ 0.001 ; ns = not significant.

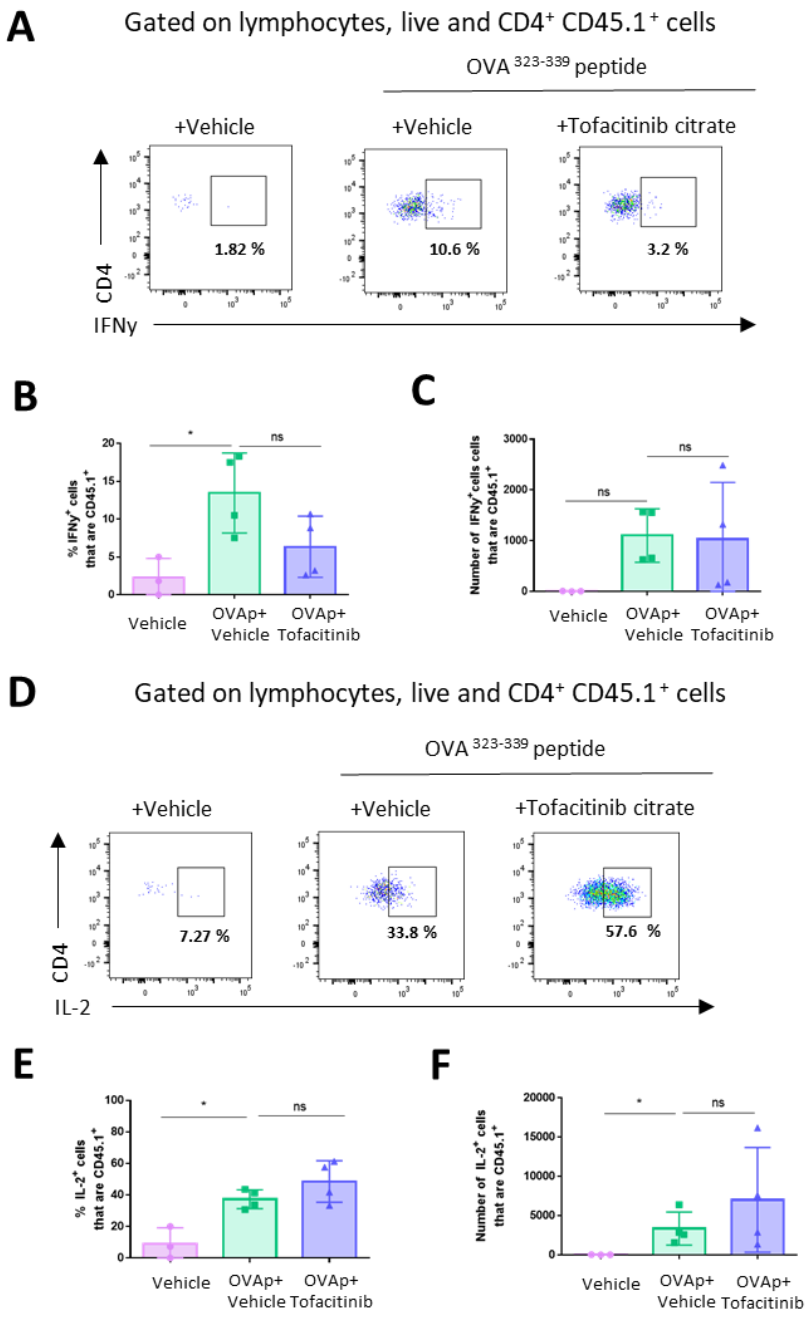


Figure 4-7. Tofacitinib citrate administration *in vivo* does not affect the capacity of adoptively transferred CD4⁺ T cells to produce IFNy or IL-2.

C57BL/6 mice received 1x10^6 OT-II CD4$^+$ T cells and 24 h later were challenged with LPS or LPS with OVA$^{323-339}$ peptide in the footpad. Mice received treatment by gavage of either vehicle or tofacitinib citrate (25 mg/kg) as described before with the last dose administered 40 minutes before sacrifice. Popliteal lymph nodes were removed, mashed into single cell suspension and half of the cells from each lymph node were stimulated with PMA and Ionomycin with addition of Brefeldin A for 4 h at 37^{o}C 5% CO_2. . Cells were then stained by FACS for CD4 and CD45.1 surface markers, followed by intracellular staining for IFNy and IL-2, and analysed by flow cytometry. Lymphocytes were gated on live cells and CD45.1$^+$ cells identified as adoptively transferred (OT-II) cells. Scatter plots represent levels of IFNy (A) and IL-2 (D) expression in CD4$^+$CD45.1$^+$ T cells. Graphs depict the mean (± SD) percentage of IFNy (B) and IL-2 (E) positive CD4$^+$CD45.1$^+$ T cells, and the mean (± SD) numbers of cells expressing respective cytokines (C and F). Results represent a single experiment with 3 mice in 'Vehicle only' group and 4 mice in the other two groups. Statistical differences between groups were assessed by One-way ANOVA and Turkey's multiple comparison test in GraphPad prism *designates a *p*-value of ≤ 0.05; ns = not significant.

4.2.6 Tofacitinib citrate treatment diminishes *in vivo* T-bet expression by endogenous CD4$^+$ T cells but does not alter their capacity for IFNy and IL-2 production.

Apart from assessing the drug impact on the transferred antigen-specific CD4$^+$ T cells, it was equally important to examine the consequences of tofacitinib treatment on the endogenous CD4$^+$ T cell function. Endogenous CD4$^+$ T cell population was easily distinguishable and comprised a comparable percentage of total popliteal lymph node cells in each of the treatment groups (Figure 4-5A). In contrast to OT-II cells, the endogenous population comprised only a small proportion of CD4$^+$ T cells expressing activation marker CD44^{hi} (Figure 4-8A,B), and this fraction of activated CD4$^+$ T cells likely consisted of mainly recirculating memory cells of the irrelevant specificities and a small number of OVA$^{323-339}$-specific T lymphocytes. However, no significant differences in the percentage of CD44^{hi} cells were observed between treatment groups (Figure 4-8B) due to a relatively low frequency of OVA$^{323-339}$-specific endogenous CD4$^+$ T cells and the associated difficulty in detecting their priming activation in response to cognate peptide.

The endogenous CD4$^+$ T cells, similarly to OT-II cells, upregulated their T-bet expression following antigenic challenge in the vehicle-treated mice, while in the animals receiving tofacitinib T-bet expression was notably lower, in both percentage and cell number, and remained at the level comparable to that in the control mouse (vehicle-only) group (Figure 4-8C,D). Mirroring the changes in T-bet expression and in line with functional changes observed in transferred CD4$^+$ T cells, the percentage and number of their IFNy-producing endogenous counterparts slightly but significantly increased in the presence of OVAp in vehicle-treated mice, while in tofacitinib-treated group IFNy production was reduced, although not significantly (Figure 4-8E,F). Interestingly, endogenous CD4$^+$ T cells notably upregulated IL-2 production upon antigenic challenge in vehicle-treated group but no alterations in IL-2 production were achieved by tofacitinib administration (Figure 4-8G,H).

Thereby, the endogenous $CD4^+$ T cells of unknown specificities in the antigen-specific inflammation model employed were presumably recruited to the draining lymph node as activated effector/memory cells. Tofacitinib treatment notably diminished the endogenous $CD4^+$ T cell ability for T-bet expression during antigenic challenge, but the capacity for IFNy production was not significantly compromised. However, since the drug-induced differences in both transferred and endogenous $CD4^+$ T cells are not statistically significant, and the activation status of the endogenous cells is not fully explored, no definitive conclusions can be made from the current comparison and further experiments would be required to assess the above possibilities.

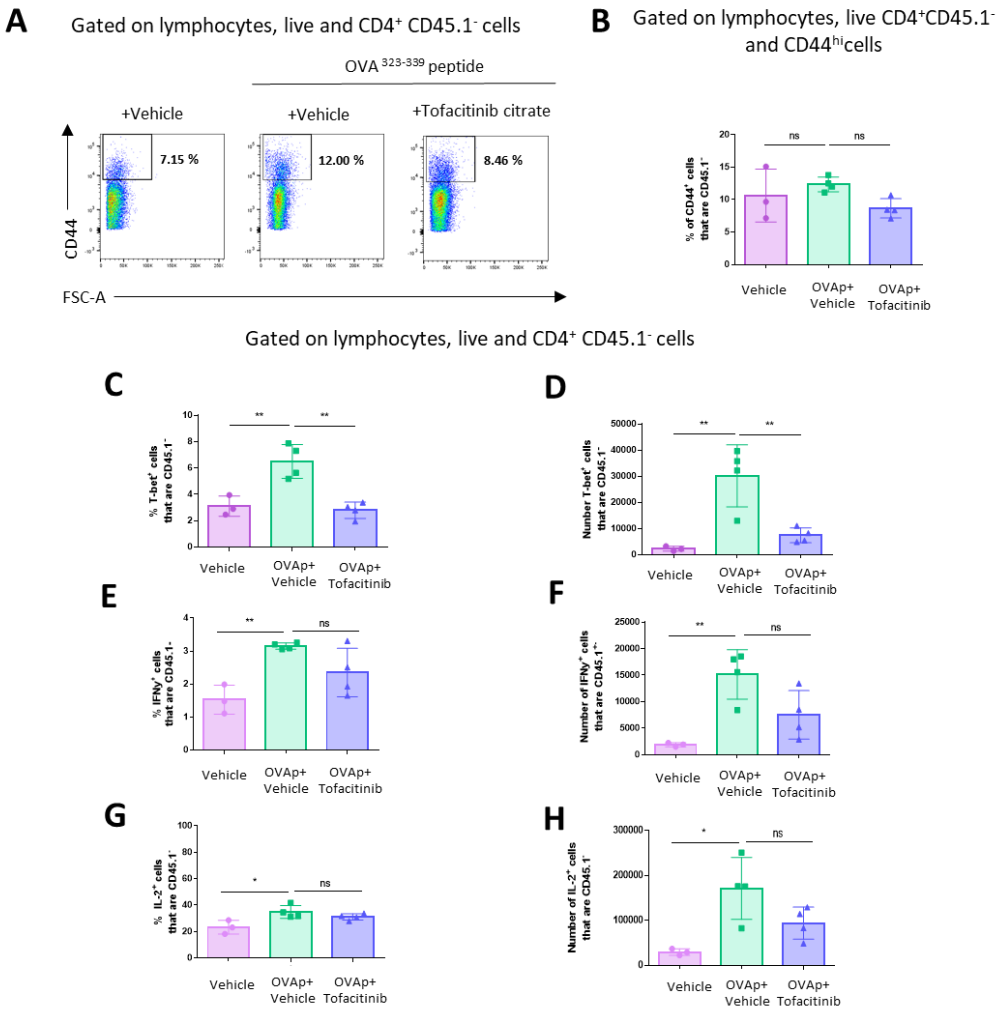


Figure 4-8. Tofacitinib citrate treatment diminishes *in vivo* T-bet expression and preserves capacity for IFNy and IL-2 production of endogenous CD4⁺ T cells in the adoptive transfer experiment.

C57BL/6 mice received 1x10^6 OT-II CD4$^+$ T cells and 24 h later were challenged with LPS or LPS with OVA$^{323-339}$ peptide in the footpad. Mice received treatment by gavage of either vehicle or tofacitinib citrate (25 mg/kg) once on the day of cell transfer and then twice daily on the consecutive 2 days. On day 3 after challenge, mice received a single treatment dose, were sacrificed 40 min later and their popliteal lymph nodes were removed and mashed into a single cell suspension. Half of the cells from each lymph node were stimulated ex vivo with PMA/Ionomycin in presence of Brefeldin A for 4 h at 37ºC 5% CO_2. All the cells were then stained by FACS for CD4, CD44, and CD45.1 surface markers, with stimulated cells stained further for intracellular cytokines IFNy and IL-2, while unstimulated fraction of lymph node stained for T-bet. Cells were subsequently analysed by flow cytometry. Lymphocytes were gated on live cells and the CD4$^+$CD45.1$^-$ cell fraction was identified as an endogenous CD4$^+$ T cell population. Endogenous CD4$^+$ T cells were assessed for CD44 (A,B) and T-bet (C-D) expression, and production of IFNy(E,F) and IL-2 (G,H) in each treatment group. Graphs depict mean ± SD of the percentage of CD44hi expressing CD4$^+$CD45.1$^-$ cells (B), percentage (C) and number (D) of T-bet-positive CD4$^+$CD45.1$^-$ cells, as well as the percentage (E and G) and number (F and H) of IFNy and IL-2-positive CD4$^+$CD45.1$^-$ cells, respectively. Results represent a single experiment with 3 mice in 'Vehicle only' group and 4 mice in the other two groups. Statistical differences between groups were assessed by One-way ANOVA and Turkey's multiple comparison test in GraphPad prism. **designates a *p*-value of ≤ 0.01; ns = not significant.

4.3 Discussion

4.3.1 Tofacitinib citrate successfully inhibits JAK/STAT pathway signalling in whole blood CD4$^+$ T cells when administered *in vivo*

Prior to investigating the effect of tofacitinib treatment *in vivo*, it was necessary to confirm that tofacitinib citrate, a drug form employed for the *in vivo* experiments, had the same mode of action as its alternative formulation, tofacitinib base. The addition of tofacitinib citrate to the *in vitro* co-cultures during OVA$^{323-339}$-specific OT-II CD4$^+$ T cell priming with peptide-pulsed dendritic cells achieved effective inhibition of cytokine signalling in CD4$^+$ T cells and reduced activation of downstream signal-transducing STAT proteins (STAT3 and STAT5). The reduced responsiveness to cytokine stimulation had resulted in impaired CD4$^+$ T cell differentiation into functional Th1 cells and manifested in diminished IFNy and enhanced IL-2 production. Since tofacitinib citrate demonstrated efficiency *in vitro* and its effect on the antigen-specific CD4$^+$ T cell function was closely comparable to that of the alternative base form, the drug was further employed for *in vivo* administration.

Tofacitinib citrate was first tested for its ability to inhibit the JAK/STAT pathway in whole blood leukocytes. Conventionally, STAT protein phosphorylation is measured at both basal state of the cells and following their stimulation with cytokines, and the difference in phosphorylation levels between the two states used to indicate the efficacy of the inhibitor. 1 hour following administration of a single tofacitinib citrate dose to C57BL/6 mice, whole blood cells, including CD4$^+$ T cells, exhibited notably smaller shifts in pSTAT3 levels upon cytokine stimulation compared to the cells from vehicle-treated mice. Interestingly, the difference in STAT3 phosphorylation levels was apparent even at the baseline level in unstimulated cells. Thus, the drug efficacy based on STAT3 phosphorylation inhibition was subsequently only assessed at the basal state of whole blood leukocytes. Importantly, a 25 mg/kg dose used in this experiment has achieved a detectable inhibition of IL-6 signalling in CD4$^+$ T cells, possibly mediated through JAK1/JAK2/pSTAT3, within 1 hour, which was consistent with

IL-6 signalling inhibition, although more complete, in $CD8^+$ T cells using 50 mg/kg drug dose.[344] Since a single oral 25 mg/kg dose achieved sufficient JAK/STAT signalling inhibition *in vivo* and sustained tofacitinib levels in plasma at half-maximal inhibitory concentration (IC_{50}) of JAK1 heterodimer for approximately 12 hours, a twice-daily oral administration of the above dose was established as a therapeutic regimen for the adoptive transfer experiment. Of note, the RA patients also receive twice daily oral tofacitinib dose, and their average plasma concentration of 100 nM was comparable to that achieved in mouse models.[344]

Tofacitinib base form, similarly to a number of newly emerging drugs, is poorly water-soluble, which does not appear to be the efficacy-limiting factor in the *in vitro* assays but is a major hindrance for achieving acceptable drug bioavailability *in vivo*. The poorly soluble compound is eliminated from the body before a therapeutically sufficient amount of it is able to dissolve and be absorbed in the gastrointestinal tract (and enter the circulation). While the dose increase could potentially overcome this issue, it would lead to other negative consequences including risk of drug toxicity, reduced patient compliance, and increased treatment costs.[521] Thus, to ensure high compound solubility, tofacitinib was synthesized in a citrate salt Form A, the only existing crystalline form of the drug, which exhibited acceptable dissolution rates across physiological pH scale. In the intestine, the salt following compound dissolution acts as a buffer for the basic drug, decreasing the pH of the microenvironment and thereby enhancing dissolution and thus an absorption of the active pharmacological compound of the drug itself.[520] The oral administration of the tofacitinib citrate salt allowed to achieve an oral bioavailability of 74%, with a peak plasma concentration reached within only 0.5-1 h post-dose. In addition to enhanced solubility and absorption, and subsequent improvement in the clinical efficacy of tofacitinib, crystallization enabled to achieve higher purity of the compound, while salt form also representing a more hydrolytically and thermally stable substance.

4.3.2 Tofacitinib citrate treatment supresses T-bet but not cytokine expression by adoptively transferred OT-II CD4$^+$ T cells during priming *in vivo*.

CD4$^+$ T cell priming represents a key step in self-tolerance breakdown and initiation of the autoimmune response seen in the initial stages of RA, as well as continuing throughout disease as they respond to the newly released self-epitopes from the damaged joint contributing to disease chronicity. Thus, therapeutic interference with CD4$^+$ T cell priming could ultimately prove beneficial at different stages of the disease. In the previous chapter tofacitinib exposure during priming *in vitro* was shown to impair CD4$^+$ T cell differentiation into Th1 helper subset, and to subsequently diminish cell capacity for subset-specific pro-inflammatory cytokine production. I thus aimed to replicate this experiment *in vivo* in the setting of antigen-specific inflammation. The use of transgenic OT-II cells, previously employed *in vitro*, allowed to specifically assess the outcome of the antigen-specific CD4$^+$ T cell priming by their cognate antigen *in vivo* in the presence of the drug, as well as separately assess the response of the endogenous CD4$^+$ T cell population to the treatment.

I have first confirmed that *in vivo* tofacitinib citrate administration in the context of antigen-specific inflammation achieved a notable reduction in circulating leukocyte pSTAT3 levels at their basal state when compared to the pSTAT3 in the antigen-challenged vehicle-treated mouse group. Thereby, since the drug was effective at inhibiting its target signalling pathway, in particular in CD4$^+$ T cells, in this T cell priming experiment, any changes observed in the lymphocyte phenotype and function could be directly attributed to the effect of tofacitinib.

The adoptively transferred OT-II CD4$^+$ T cells were retrieved from the popliteal lymph nodes 3 days after recipient mice received antigenic challenged and twice-daily oral tofacitinib treatment. While these OVA peptide-specific cells demonstrated successful clonal expansion and activation regardless of drug exposure, the percentage and number of these cells expressing transcription factor T-bet was markedly lower in the drug-treated animal group. T-bet

expression is crucial for terminal $CD4^+$ T cell commitment towards the Th1 subset, and its transcription initially requires IFNγ STAT1-dependent signalling during T cell priming. Upon T-bet expression, the transcription factor directly enhances IFNγ production through *Ifng* locus remodelling and induces IL-12Rß2 expression, thereby further promoting its own expression and establishing an IFNγ self-enhancing loop.[515,522] Tofacitinib likely interferes with OT-II $CD4^+$ cell polarization towards a mature Th1 subset by inhibiting signalling through their IFNγ receptor. Exposure to drug throughout $CD4^+$ cell priming *in vitro* resulted in the closely comparable outcome, with the drug mechanism preventing Th1 differentiation likely being the same both *in vitro* and *in vivo*.

Despite the diminished potential of antigen-specific $CD4^+$ cells for Th1 polarization following tofacitinib exposure, their capacity for subset-specific cytokine IFNγ production was not significantly impaired. Further investigation is required, possibly by assessing the IFNγ production of these cells at the later time point (day 5 after antigenic challenge), to determine if there might be a delay between the tofacitinib impact on T-bet expression and the associated notable negative effect on IFNγ production.

In line with changes seen in IFNγ production, tofacitinib treatment promoted increased IL-2 production in transferred $CD4^+$ cells *in vivo* which was not statistically significant but closely replicated the changes observed in the *in vitro* co-culture setting. In both cases, the drug might interfere with the STAT5-dependent IL-2-mediated negative feedback loop,[509] leading to the impaired inhibition of IL-2 production together with diminished IL-2Rα expression and reduced cytokine consumption. While the diminished responsiveness to IL-2 stimulation did not appear to affect OT-II T cell proliferation, it could have contributed to the impaired cell capacity for Th1 differentiation, which is also partly regulated by IL-2.[511] However, it is unclear why the *in vivo* drug administration did not achieve a substantial change in cell ability for IL-2 production.

While the variation in above cytokine data does not allow to determine with certainty if the production of IFNγ is diminished and of IL-2 is enhanced in response to tofacitinib *in vivo*, the existing literature suggests this to hold true. A study investigating the impact of tofacitinib on a mouse model of acute graft-

versus-host disease (GvHD) demonstrated that despite the preserved ability for proliferation, the number of donor $CD4^+$ T cells producing IFNy had significantly decreased in all lymphoid organs[516]. As this effect was observed during the early phase of GvHD induction, and the naïve $CD4^+$ T cells are known to be among the initial responders post-transplantation[523], these results could be closely correlated with our findings, despite being obtained in a different inflammatory context. Another study demonstrated diminished IFNy production following tofacitinib treatment in the joints of SKG mice spontaneously developing chronic arthritis, which, along with other parameters, correlated with long-term clinical improvement.[312] However, $CD4^+$ T cells in the inflamed joint would represent a heterogenous population at different activation states, thus the effect could not be directly attributed to the drug impact on $CD4^+$ T cell priming alone.

There could be several possibilities explaining the discrepancy between the cytokine data in the adoptive transfer experiment and the *in vitro* setting. One potential explanation could be a more complex drug biodistribution *in vivo*, which might be different for specific tissues, as well as its availability for the target cells, which are numerous in both circulation and tissue sites of interest. Moreover, since the oral twice daily tofacitinib administration results in noticeable fluctuations of its average plasma concentrations over time, the drug bioavailability is also not continuously stable and thus a longer time period might be required to achieve sufficient cytokine signalling inhibition in the target cells. Therefore, since the OT-II cell potential for Th1 differentiation was notably reduced and strongly correlated with cytokine production capacity (as seen *in vitro*), continuing the drug treatment and assessing cell function at the later time point (day 5 after antigenic challenge) might produce more consistent and clearer results. The other possibility is insufficient animal numbers in the treatment groups and employing more mice (n=5-6) for each group might help to reduce the variability of obtained values and obtain more clear statistical trends. Finally, there could be a failure to maintain a sufficient average tofacitinib plasma concentration over the course of the experiment, which, given the rapid reversibility of JAK inhibition, might make the target cells more responsive to the cytokine stimulation. The possible solution might include increasing the total daily drug dose or employing the osmotic mini- pump for more consistent drug administration.

Although not explored experimentally in this chapter, tofacitinib could also influence the priming outcome by affecting dendritic cell function. An initial report by Rivas-Caicedo *et al.* described bone marrow-derived dendritic cells from Jak3$^{-/-}$ mice as having diminished CCR7-mediated homing to lymph nodes *in vivo* combined with failures in co-stimulatory molecule CD80/86 and MHC-II upregulation, collectively resulting in the reduced capacity of Jak3-deficient BMDCs to efficiently promote T lymphocyte proliferative responses.[524] Another study conducted by Kubo and colleagues employed tofacitinib, as a potent JAK3 inhibitor, to similarly assess its impact on human monocyte-derived dendritic cell (moDC) maturation and effector function. While MHC-II expression remained unaffected, *in vitro* exposure to tofacitinib during LPS stimulation significantly supressed CD80/86 expression alongside reduced pro-inflammatory TNFα, IL-1β and IL-6 production. In addition, the drug pre-treated moDCs showed decreased capacity for naïve CD4^{+} T cell stimulation, leading to diminished T lymphocyte proliferation and IFNγ production.[309] In a more recent *in vivo* study, mouse BMDCs exposed to tofacitinib prior to LPS stimulation, loaded with disease-specific antigen and adoptively transferred into an EAE mouse model not only exhibited comparable phenotypic and functional alterations but also demonstrated reduced IL-12 and IL-23 production. Importantly, this resulted in impaired differentiation towards Th1/Th17 subsets in favor of the CD25^{+}Foxp3^{+} Treg population expansion.[514] Collectively, dendritic cell exposure to tofacitinib prior to or during their maturation appears to impair their functional capacity and thereby compromises their ability to provide sufficient ‘signal 2’(co-stimulation) and ‘signal 3’ (polarizing cytokines) to naïve CD4^{+} T cells, crucial for effective T lymphocyte activation and polarization. Such a tolerogenic dendritic cell phenotype was currently only induced *in vitro,* thus it would be important to investigate if the *in vivo* administration of the drug would induce a similar functional profile in the endogenous dendritic cells. Since in my adoptive transfer experiment tofacitinib treatment is initiated the day before administration of LPS and hence dendritic cell maturation induction, it could be a suitable context to assess the functional changes in the endogenous dendritic cells, which could in turn substantially contribute to impaired adoptively transferred CD4^{+} T cell differentiation and function.

4.3.3 Tofacitinib citrate administration *in vivo* diminished T-bet expression by endogenous CD4$^+$ T cells but did not impact cytokine production following non-specific activation.

Due to the nature of the adoptive transfer system, I was also able to distinguish the endogenous polyclonal CD45.1$^-$ CD4$^+$ T cell population in the popliteal lymph nodes and assess their capacity for differentiation and cytokine production. Since there was no increase observed in the percentage of CD44$^+$ cells following antigenic challenge, but a significant increase in T-bet expression was detected in a small proportion of endogenous CD4$^+$ T cells, I proposed that these cells did not undergo antigen-specific priming but rather represented previously activated cells recruited in response to inflammation. Interestingly, the *in vivo* tofacitinib administration significantly supressed T-bet expression in these bystander CD4$^+$ T cells, while preserving their capacity for IFNy and IL-2 production. A comparable trend was observed in the adoptively transferred antigen-specific CD4$^+$ T cells, and it is unclear whether the non-significant changes in cytokine production capacity in both cell populations are due to an early experiment termination or other possible experimental issues, or represent a true outcome of the drug treatment. An *in vitro* study investigating tofacitinib effect on bystander CD4$^+$ T cells co-cultured differentially labelled PHA-stimulated and non-stimulated PBMCs from healthy donors and observed profound activation and proliferation of the non-stimulated cells activated in the mixed culture in a bystander fashion. Addition of tofacitinib to the co-culture notably impaired bystander T lymphocyte ability for IFNy production while enhancing IL-2 generation.[485] Thereby, at least in an *in vitro* setting tofacitinib appears to diminish the pro-inflammatory function of cytokine-activated bystander T cells.

Bystander activation does not require TCR engagement, thus the cells can be activated by cytokine stimulation (and possibly co-stimulation) when recruited to the cytokine-rich inflammatory site alongside antigen-specific auto-reactive T cells. Effector and memory T cells, in particular, were found to have a lowered threshold for such bystander activation. When activated, these cells can induce TNFα secretion by monocytes, either indirectly or through cell-cell contact,

thereby sustaining and perpetuating the cycle of chronic inflammation.[482] Thus, the ability to block cytokine signalling might enable tofacitinib to also inhibit bystander T cell activation and thereby possibly limit the disease progression through yet another immunomodulatory mechanism. Our experimental model could be utilized to further elucidate the bystander T cell fate following tofacitinib exposure *in vivo*, with a more detailed assessment to confirm bystander T cell phenotype and possibly their function in the co-culture with monocytes.

Overall, in this chapter, by using the adoptive transfer of $OVA^{323-339}$-specific OT-II $CD4^+$ T cells to elicit antigen-specific inflammation *in vivo,* I have demonstrated that tofacitinib treatment throughout the priming of $CD4^+$ T cells impairs their ability for differentiation into Th1 cells and reduces subset-specific IFNy production, with the latte r not reaching statistical significance. The antigen-experienced endogenous $CD4^+$ T cells similarly exhibited supressed T-bet expression but retained their ability for IFNy production. Upon demonstrating the immunomodulatory capacity of tofacitinib in an *in vivo* setting, the next step was to assess whether this drug impact on $CD4^+$ T cells is achievable in a disease mouse model and whether tofacitinib exhibits any therapeutic efficacy in this setting.

5 Assessment of tofacitinib treatment effect on $CD4^+$ T cell behaviour and joint pathology in the 'breach-of-tolerance' mouse model of early RA

All animal experiments were done with help from Robert Benson. Samples for the histological assessment were prepared by Lynn Stevenson. Joint tissue sections were scored for the signs of pathology by Robert Benson.

5.1 Introduction

My results have thus far demonstrated that the presence of tofacitinib during naïve $CD4^+$ T cell priming resulted in the diminished cell ability for differentiation towards Th1 phenotype and the associated abrogation of IFNy production capacity, both in the *in vitro* setting and in the adoptive transfer model (*in vivo).* In contrast, the antigen-experienced cells appeared to retain their functional capacity when exposed to tofacitinib during their re-activation *in vitro*. I have therefore decided to proceed by assessing the effect of tofacitinib treatment in a murine model of RA to gain further understanding of immunomodulatory drug mechanisms.

Multiple Phase III clinical trials ranging from 6 months (ORAL trials)[525] up to 9.5 years (ORAL Sequel long-term extension study)[526] have previously evaluated tofacitinib for clinical efficacy, impact on structural disease progression and adverse effect profile. Across the trials, tofacitinib demonstrated therapeutic efficacy in both early and late active-phase disease and in both treatment-naïve and non-responder patients. Significant drug efficacy was observed equally with tofacitinib used as a monotherapy or in combination with methotrexate and other conventional synthetic DMARDS (csDMARDs), and, importantly, tofacitinib was effective in patients with active RA failing to respond to several biologic DMARDS with distinct modes of action. Clinically relevant end-points, including ACR20, ACR50 and ACR70, DAS28 score, physical function improvement (HAQ-DI) and pain were notably improved, with some of the improvements distinguishable as early as 2 weeks following treatment induction[525]. The structural joint damage also showed minimal progression in the majority of patients.[527] However, while these and other clinical parameters manifest rapid and persistent tofacitinib efficacy in the clinic, there is a lack of understanding of biological (immunological) mechanisms underlying the observed physiological responses to the drug.

Multiple rodent models of arthritis were thus employed for in-depth investigation of tofacitinib treatment impact on systemic inflammation, immune cell behaviour and associated joint pathology. The disease models of both

induced (collagen-induced arthritis, CIA; adjuvant-induced arthritis, AIA) and spontaneous (SKG mice) chronic arthritis were used, which closely recapitulate clinical and histological features of the advanced human disease phase. Clinical scores indicating the disease severity were markedly reduced in arthritic mice receiving tofacitinib after initial 2-3 days of the treatment[305,311], remained supressed throughout the duration of the study[305,311,312] and, in the SKG mouse model, this effect persisted for another 3 weeks following drug withdrawal[312]. Such rapid disease amelioration could be associated with the drug-induced decrease of inflammatory mediator concentration in both plasma and paw tissue, with a significant reduction in IL-6, IL-17, CCL2 (MCP-1) and CXCL10 (IP-10) observed as early as 4 hours post-dose.[305,310] Histopathological analysis revealed tofacitinib administration to abolish synovial hypertrophy and notably limit inflammatory cell influx in both surrounding synovial tissue and joint cavity[305,311,312]. In line with that, in the SCID-HuRAg mice bearing human synovium and cartilage implant synovial invasion into cartilage was supressed in the tofacitinib-treated animals.[306] Cartilage and bone erosions were also markedly limited by the drug, with the bone-degrading osteoclasts largely absent from the bone cavity of the tofacitinib-treated AIA rats.[310,311] Finally, SKG mice exhibited reduced IFNy, IL-17 and increased IL-10 levels in the joint tissue following drug exposure, suggesting a possible correlation between pathology improvement and suppression of Th1 and Th17 cell pro-inflammatory function.[312] Interestingly, LaBranche *et al*. also demonstrated decrease T cell production of RANKL, which promotes osteoclast differentiation and activation[310], thus highlighting an additional possible contribution of T cells to promoting disease pathogenesis, and further demonstrating the importance of their in-depth functional assessment in the context of experimental arthritis.

The above animal studies confirm the tofacitinib treatment to promote the rapid and effective amelioration of the disease at its active state. This comes in line with the strategy of clinical RA management currently recommending the use of JAK inhibitors predominantly as the third-line treatment ,following the inadequate response or intolerance to one (or several) biologic DMARDs, thus introducing JAK inhibitors fairly late in the disease course.[141] Considering the ongoing trend for therapeutic intervention at the onset of RA, allowing to prevent the joint damage and even achieve remission, it would be of interest to

determine if JAK inhibitors demonstrate similar efficacy early as the first-line therapeutic modality.

I have therefore employed a mouse model of acute transient RA developed in our laboratory, which displays a number of features characteristic of pre-articular/pre-clinical phase of RA. The model exhibits self-tolerance breakdown to joint-specific antigens, induced by the adoptive transfer of transgenic Th1-polarized $CD4^+$ T cells of joint-irrelevant antigen specificity.[455] Unlike the CIA model, where the breach of self-tolerance is achieved by external administration of the self-antigen in a very strong adjuvant, in our model this event occurs in endogenous T cells following the establishment of acute localised inflammation driven by a transferred Th1 cell population of joint-irrelevant specificity. Apart from developing measurable autoantibody response, the model, although being transient/self-limiting, also exhibits prominent histological changes (inflammatory cell influx, synovial hyperplasia, hypervascularization, cartilage and bone degradation) characteristic of human early stage/preclinical disease. In addition, the current model allows monitoring fates/functions of both inciting transgenic $CD4^+$ T cells and their endogenous counterparts in the context of antigen-specific priming and/or re-activation in the environment of the developing pathology.

The previous studies of the acute RA mouse model revealed that the inflammatory response to the non-specific inflammatory mediator (LPS) in the joint also resulted in the recruitment of (OT-II) T cells of joint-irrelevant specificity and the (associated) development of some aspects of joint pathology. However, while the comparable (histo)pathological features were observed in the HAO-challenged mice, the antigen-specific activation of these T cells of irrelevant specificity appeared to be a prerequisite for the breach of self-tolerance and autoantibody response generation.[528] A more recent study have also dissected the dynamics of $CD4^+$ T cell recruitment to the joint, demonstrating that the recruitment of inciting antigen-specific (OT-II) T cells occurred alongside the accumulation of oligoclonal $CD4^+$ T cell population exhibiting a pro-inflammatory phenotype, with a large proportion of these cells interacting with $CD11c^+$ DCs in the manner indicative of cognate antigen recognition and thus likely representing an autoreactive cell population.

Importantly, the adoptively transferred Th1 $CD4^+$ T cells of irrelevant specificity ($E\alpha_{52-68}$ peptide-specific) were also found to be able to infiltrate the arthritic joint even in the absence of their cognate antigen.[468] The acute RA model also allowed to establish a novel mode of action of abatacept, occurring through supression of follicular T cell phenotype acquisition in antigen-specific T cells and abrogation of their subsequent migration to the B cell follicles. Abatacept also inhibited antigen-specific (OT-II) T cell activation and proliferation, and its cumulative effects resulted in the prevention of self-tolerance breakdown, diminished autoantibody responses, and improvement in joint pathology[529].

Thus, in this chapter I employed the 'breach-of-tolerance' mouse model of pre-clinical RA to address the following:

- To assess the capacity of adoptively transferred Th1 cells for T-bet expression and IFNy production upon their re-activation *in vivo* in the presence of tofacitinib

- To determine endogenous $CD4^+$ T cell capacity for T-bet expression and IFNy production after the exposure to tofacitinib *in vivo*

- To investigate whether tofacitinib treatment can interfere with the breakdown of self-tolerance by supressing autoantibody response

- To address the ability of tofacitinib to ameliorate the inflammation and joint pathology when administered at the early stages of RA development

5.2 Results

5.2.1 Tofacitinib has no impact on T-bet expression or IFNγ production by adoptively transferred OT-II CD4$^+$ Th1 cells in early RA mouse model

To generate the experimental RA mouse model, I have adopted the original protocol developed in our laboratory by Maffia *et al.*[455] (Figure 5-1) First, CD4$^+$ T cells were isolated from lymph nodes and spleens of transgenic OT-II mice, and their polarization towards Th1 subset was achieved by co-culturing them with splenocytes pulsed with OVA$^{323-339}$ peptide in the presence of Th1 polarizing factor/cytokine IL-12 and anti-IL-4 antibody to limit skewing towards Th2 phenotype. After 3 days in polarizing culture, the percentage of OT-II CD4$^+$ T cells expressing Vα2Vβ5 TCR was determined by flow cytometry and a total of 2x10^6 OT-II cells were adoptively transferred (via the tail vein) into all C57BL/6 recipient mice. It has been previously confirmed the OVA$^{323-339}$-specific Th1 cells polarized in this manner to produce high levels of signature cytokine IFNγ along with negligible levels of IL-5.[455] One day following the adoptive transfer, all recipient mice were immunized subcutaneously (scruff) with the whole OVA protein emulsified in complete Freund's adjuvant (CFA) to promote OT-II CD4$^+$ T cell activation and clonal expansion in the peripheral draining lymph nodes. Following 10 days after immunization, mice received heat-aggregated OVA (HAO) challenge into the hind limb footpads, proximal to the ankle joints, and the control animals were challenged with PBS instead. HAO injection was aimed to direct the migration of OT-II CD4$^+$ T cells into the ankle (tarsal) joints and associated popliteal lymph nodes where they can exert an inflammatory response to their cognate antigen and promote the favourable conditions for the breakdown of self-tolerance to occur. Tofacitinib treatment was initiated 1 day before HAO challenge in order to saturate the system with the drug prior to the pathology induction and was thereafter administered twice daily by oral gavage at 25 mg/kg/dose, replicating the treatment regimen employed in the *in vivo* model (Chapter 4). PBS-treated and arthritic (HAO-challenged) control mouse group were treated with 0.5% DMSO in vehicle solution. On day 7 post-HAO challenge, mice received the last dose of either drug or DMSO and were

sacrificed within 1 hour. Day 7 post-HAO challenge appeared optimal for experiment termination, as the arthritic animals at this stage have a low but detectable number of adoptively transferred $CD4^+$ T cells in the (peripheral) draining lymph nodes, exhibit notable anti-OVA antibody response and begin to develop auto-antibody response against collagen II, an abundant articular antigen[455]. In addition, signs of joint pathology are evident at day 7 post-HAO injection.[455,530]

The first aim was to assess the capacity of Th1-polazired $OVA^{323-339}$-specific $CD4^+$ T cells for T-bet expression and pro-inflammatory cytokine production upon their re-activation *in vivo*. Prior to addressing the impact of tofacitinib on the (pathology-inciting) $CD4^+$ T cell function, it was crucial to confirm the drug capacity for its target pathway inhibition in our disease model setting. I have demonstrated earlier (Chapter 4) that following 3 days of treatment there was a reduction in basal STAT3 phosphorylation levels in the circulating leukocytes *in vivo*. Thus, for this experiment, the whole blood samples were collected from all recipient mice three days after HAO challenge, pulled together for each treatment group and stained by Phospho-Flow cytometry for the levels of pSTAT3 protein. As expected, mice receiving HAO injection together with a vehicle had a significant increase in the proportion of pSTAT3-positive circulating $CD4^-$ cells (30.4%) compared to those in the PBS-injected mice (2.99%)(Figure 5-2A). However, tofacitinib exposure resulted in the pSTAT3 levels in the $CD4^-$ cell population to be retained close to the baseline (3.10%). These changes were reflected in the histogram depicting pSTAT3 fluorescence intensity in the $CD4^-$ cell compartment (Figure 5-2B). In the $CD4^+$ cell compartment, the proportion of pSTAT3-positive cells in the arthritic vehicle-treated animal group (1.46%) appears to be lower than that in the non-arthritic animals (2.06%), while the tofacitinib exposure appeared to increase the percentage of pSTAT3-expressing $CD4^+$ cells (2.62%) compared to the other two animal groups. However, the evaluation of mean fluorescence intensity (MFI) for pSTAT3 in the whole $CD4^+$ T cell population (i.e. both upper left and right quadrants of the plots in Figure 5-2A) demonstrated the pronounced increase in pSTAT3 MFI in arthritic vehicle-treated group (408) from its background levels in non-arthritic (control) group (253), while the tofacitinib treatment retained the pSTAT3 levels below the baseline (239)(Figure 5-2, table).In line with that, a

clear positive peak was evident in a pSTAT3 histogram for the $CD4^+$ cell population from arthritic vehicle-treated mice, which was absent in the drug-treated group (Figure 5-2B). Thus, I have confirmed the capacity of tofacitinib to inhibit the phosphorylation of STAT3 protein in both $CD4^+$ and $CD4^-$ circulating cell populations and to ,thereby, efficiently interfere with signaling through its target pathway in the RA mouse model/in vivo.

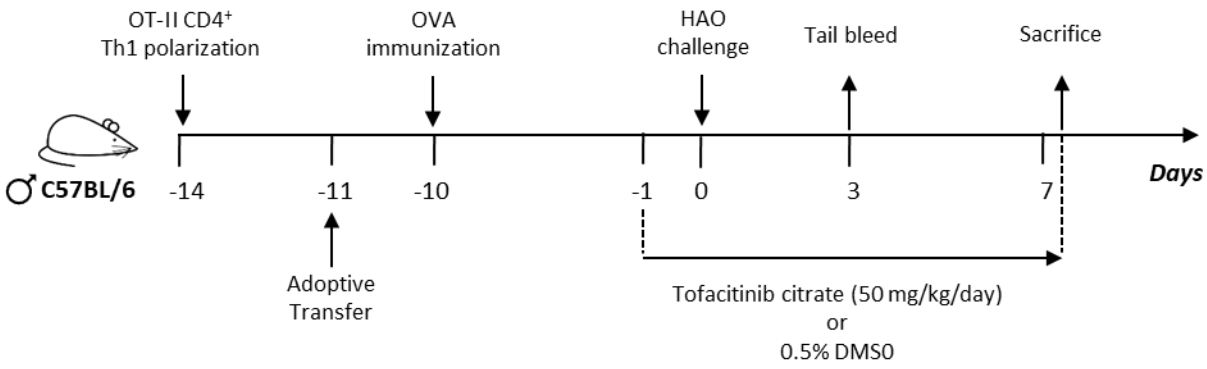
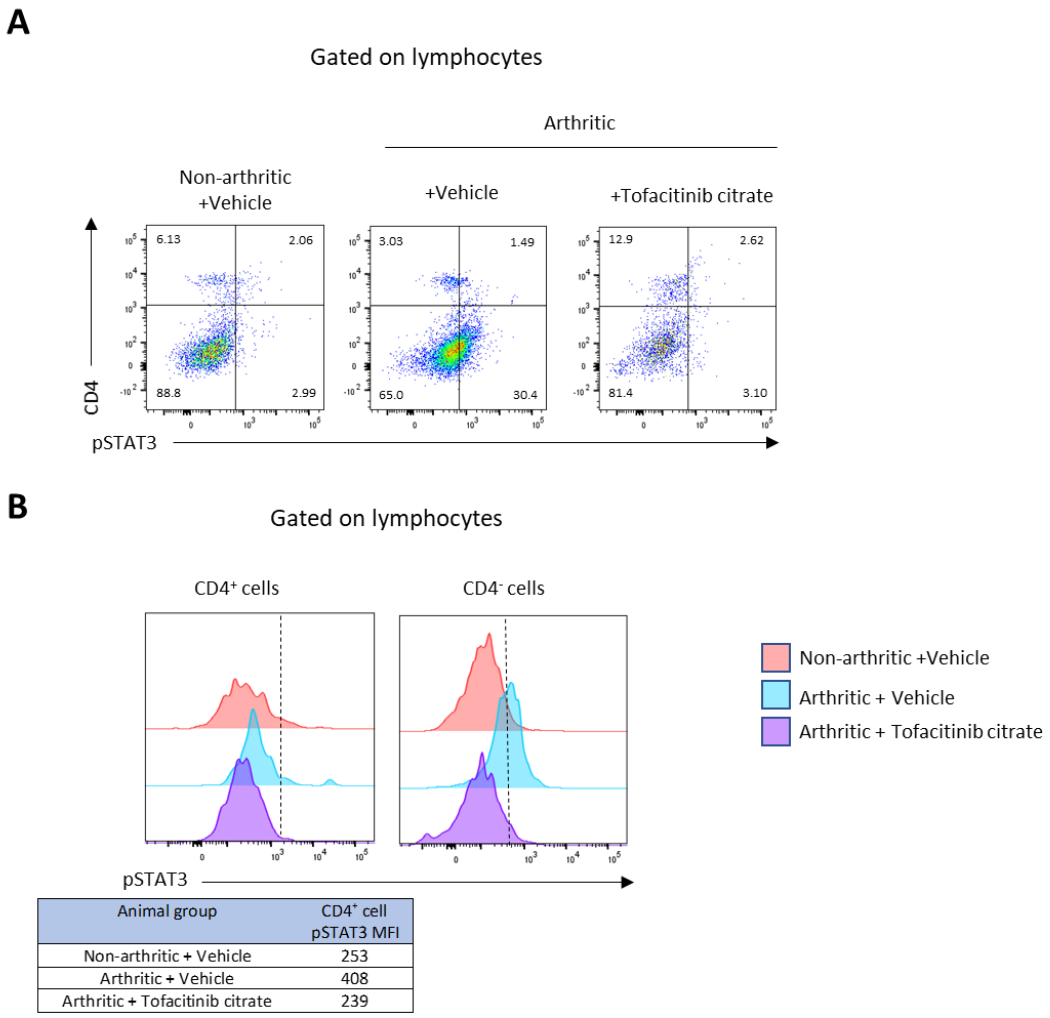


Figure 5-1. Experimental protocol of ovalbumin (OVA)-mediated early RA mouse model induction and tofacitinib administration.

$CD4^+$ T cells were isolated from lymph nodes and spleens of OT-II mice using MACS magnetic isolation kit. The Th1 polarization was induced by co-culturing $CD4^+$ T cells with splenocytes in the presence of $OVA^{323-339}$ peptide(1 ug/ml), IL-12 (10 ng/ml) and anti-IL-4 antibody (2 ug/ml) in 75 cm^2 culture flasks at 37°C 5% CO_2 for 72 h. The proportion of $CD4^+$ $V\alpha2^+V\beta5^+$ OT-II cells was determined by flow cytometry and a total of $2x10^6$ Th1 OT-II cells were injected intravenously (tail vein) into all C56BL/6 recipient mice. One day following the adoptive transfer, all recipients were immunized subcutaneously (scruff) with 100 ug of OVA protein in complete Freund's adjuvant (CFA), and 10 days later were challenged with subcutaneous injection with 100 ug of heat-aggregated OVA (HAO) into both hind limbs, close to the ankle joints. Control animals received a PBS injection instead. Starting one day before the HAO challenge, HAO-treated mouse group received tofacitinib citrate treatment twice daily (25/mg/kg/dose) by oral gavage. Another HAO-treated group and (PBS-treated) control groups received 0.5% DMSO in vehicle solution twice daily by gavage. On day 3 post-HAO challenge, blood samples were collected by the tail vein bleed for assessment of pSTAT levels by Phospho-Flow cytometry. On day 7 after the HAO challenge, mice received the last dose of either drug or DMSO and were sacrificed within 1 h. After the sacrifice, popliteal lymph nodes were collected for $CD4^+$ T cell analysis by FACS, hind limbs were used for histological assessment and the whole blood was processed for antibody detection in the serum.



Animal group	$CD4^+$ cell pSTAT3 MFI
Non-arthritic + Vehicle	253
Arthritic + Vehicle	408
Arthritic + Tofacitinib citrate	239

Figure 5-2. Tofacitinib treatment reduces basal levels of STAT3 phosphorylation in both $CD4^+$ and $CD4^-$ circulating leukocytes of early RA mouse model.

Experimental arthritis was induced as described (Fig.5-1). Starting one day before secondary challenge with HAO, mice received tofacitinib citrate treatment twice daily (25 mg/kg/) by oral gavage. Another HAO-challenged group and control (PBS) group received vehicle solution (containing 0.5% DMSO) twice daily by oral gavage. 3 days following HAO challenge, all mice were bled from the tail vein and the blood samples collected were pulled for each treatment group. The samples were incubated with a surface antibody cocktail for 15 min at 37°C 5% CO_2. Cells were then immediately fixed, permeabilized and stained intracellularly for phosphorylated STAT3. Lymphocytes were gated based on FSC and SSC and analysed for their expression of CD4 and pSTAT3. (A) Flow cytometric analysis of whole blood cells, with scatter plots representing pSTAT3 levels in the whole blood from mice from each treatment group. The numbers indicate percentage on cells in each quadrant, with the percentage of $CD4^+$ and $CD4^-$ cells expressing pSTAT3 shown in the top right and bottom right quadrants, respectively. (B) Histograms show levels of pSTAT3 from scatter plots in (A) individually for $CD4^+$ and $CD4^-$ cells. The table shows the mean fluorescent intensity (MFI) for pSTAT3 in a total $CD4^+$ cell population from each treatment group. Results represent a single experiment with 5 mice in each treatment group. Dashed lines represent the ‘ approximate threshold for STAT phosphorylation from the baseline. ‘Non-arthritic’ designates a control group receiving PBS injection as a secondary challenge. Mice from ‘arthritic+ vehicle’ and ‘arthritic+ tofacitinib citrate’ groups were challenged with HAO and developed signs of early arthritis.

Upon confirming the effective JAK/STAT pathway inhibition in circulating leukocytes in the disease model, I next sought to determine the effect of the drug on the ability of Th1-polazired $OVA^{323-339}$-specific $CD4^+$ T cells for T-bet expression and pro-inflammatory cytokine production. All cell-recipient mice were immunized with OVA/CFA 24 h following adoptive transfer, and 10 days later received peri-articular challenge with HAO in both hind limb ankle joints, as described previously (Section 5.2.1 and Figure 5-1). Mice were treated with a single tofacitinib dose (25 mg/kg) a day before HAO challenge and then twice daily for 7 days, with the final dose administered 1 h before sacrifice. While at this stage of the experimental pathology detectable population of transferred OT-II $CD4^+$ T cells are found to be recruited to the ankle joints, a substantial population of those cells could still be identified in the popliteal lymph node draining the joint[468]. Popliteal lymph nodes, draining the foot, were harvested, processed into a single cell suspension and half of the cells from each lymph node stained either for T-bet expression analysis or for the assessment of intracellular cytokine production by flow cytometry. Distinguishable populations of transferred OT-II ($CD45.1^+$) and endogenous ($CD45.1^-$) $CD4^+$ T cells were observed in all three treatment groups (Figure 5-3A). The potential clonal expansion of OT-II $CD4^+$ T cells (in response to HAO challenge) was assessed by establishing the percentage of $CD4^+CD44^{hi}$ lymphocytes which were $CD45.1^+$ (Figure 5-3B). No significant differences were observed in the percentage of adoptively transferred $CD45.1^+$ T cells between non-arthritic and arthritic (HAO-challenged) mice of either vehicle- or drug-treated groups (Figure 5-3B). However, considering the late time-point of the experiment (day 7 post-HAO challenge), it is likely that the activated and expanded OT-II $CD4^+$ T cells would have already migrated into the joint, thus it might not be feasible to detect clonal expansion within the draining lymph nodes.

Since I have previously demonstrated tofacitinib to diminish T-bet expression in the activated $CD4^+$ T cell following secondary antigenic challenge *in vitro* (Chapter 3), I sought to determine if the drug could have a comparable effect on antigen-experienced OT-II Th1 cells in the disease setting (*in vivo*). Surprisingly, T-bet expression in Th1-polazired OT-II cell population was very low among all treatment groups and did not appear to be upregulated in response to HAO immunization (Figure 5-3C,D). This was an unexpected result, as I earlier

demonstrated T-bet to be upregulated in 50-80% of previously activated OT-II cells *in vitro*, and the $CD4^+CD45.1^+$ T cells primed *in vivo* showed T-bet upregulation in at least 60% of the cell population. One possible explanation could be the failure of the OT-II cells to respond to the HAO stimulus. Alternatively, the failure to detect the re-activated $CD45.1^+$ T cell population might be due to a choice of a late time point (day 7 post-challenge). Hence, assessment of the $CD45.1^+$ T cells 2-3 days post-HAO challenge, when the maximal T cell accumulation in the lymph nodes is known to occur, might allow to better investigate both cell activation (T-bet upregulation) and the potential impact of tofacitinib treatment. In addition, OT-II T cells in this model are known to also accumulate in the joint with their highest levels detected between days 4-7 post HAO-challenge, and a proportion of these T cells was shown to undergo interactions with dendritic cells suggesting $CD45.1^+$ T cell antigen-specific activation directly in the joint tissue.[468] Thus, assessment of the OT-II cells in the joint alongside the lymph node might enable to better assess cell response to the antigen (in the context of T-bet re-expression) and its possible alterations in response to tofacitinib.

As the assessment of the drug impact on T-bet expression was inconclusive, I next investigated the capacity of OT-II T cells for IFNγ production after they had been repeatedly challenged with their cognate antigen *in vivo*. As demonstrated by the intracellular cytokine staining, the percentage of IFNγ-positive cells in the arthritic vehicle-treated group was slightly elevated compared to other treatment groups but did not reach statistical significance (Figure 5-3E,F). Importantly, the exposure to tofacitinib did not appear to notably affect the proportion of $CD45.1^+$ T cells capable of IFNγ production. This result might replicate the earlier observations in the *in vitro* setting (Chapter 3), where previously activated $CD4^+$ T cells re-challenged in the presence of tofacitinib retained their ability for IFNγ production.

Overall, the above data was inconclusive regarding the impact of tofacitinib on T-bet re-expression in antigen-experienced OT-II cells following cognate antigenic challenge. However, I have demonstrated the preserved capacity of these cells, re-activated in the presence of tofacitinib, for IFNγ production in the setting of acute experimental RA.

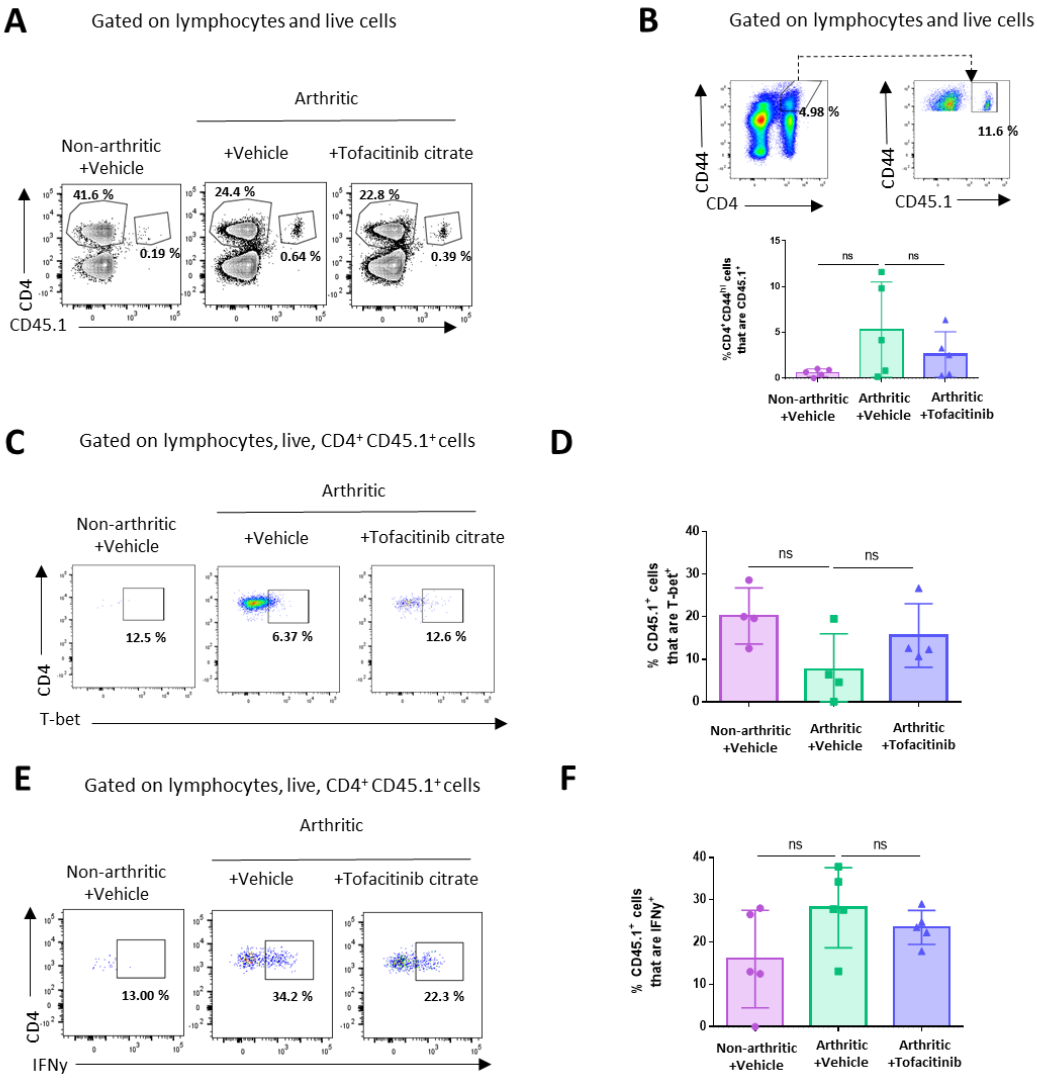


Figure 5-3. Adoptively transferred OT-II CD4⁺ Th1 cells exhibit no changes in T-bet expression or capacity to produce IFNy upon repeated antigenic challenge in the presence of tofacitinib.

Experimental arthritis was induced in mice as described before (Fig.5-1). Starting one day before a secondary challenge with HAO, mice received tofacitinib citrate treatment twice daily (25 mg/kg/) by oral gavage. Another HAO-challenged group and control (PBS) group received vehicle solution (containing 0.5% DMSO) twice daily by oral gavage. 7 days following HAO challenge mice were sacrificed and popliteal lymph nodes were collected for analysis by flow cytometry. Lymph nodes were mashed into a single cell suspension and half of the cells from each lymph node were stimulated with PMA and Ionomycin with the addition of Brefeldin A for 4 h at 37ºC 5% CO_2, followed by intracellular staining for IFNy. Another half of the cells were stained for transcription factor T-bet. Both panels included surface staining for CD4, CD44, and CD45.1 surface markers. Lymphocytes were gated on live cells and adoptively transferred and endogenous cells were identified as $CD4^+CD45.1^+$ and $CD4^+CD45.1^-$ cell populations, respectively (A). To measure the clonal expansion of adoptively transferred OT-II cells, a $CD4^+CD44^{hi}$ population was assessed for percentage of $CD45.1^+$ lymphocytes (B). The total $CD4^+CD45.1^+$ cell population was assessed for percentage of T-bet expressing (D) and IFNy secreting (F) cells, with respective scatter plots for each marker depicter in (C) and (E). Graphs depict the mean percentage ± SD, with n=4 per group for T-bet (a sample from each group was dismissed from analysis due to contamination with T-bet expressing Th1 cells used as positive control) and n=5 per group for IFNy assessment. The data represents results from a single experiment. Statistical differences between groups were assessed by One-way ANOVA and Turkey's multiple comparison test in GraphPad prism. **designates a *p*-value of ≤ 0.01. ns=non-significant

5.2.2 Endogenous CD4$^+$ T cells exhibit no changes in T-bet expression or IFNγ production in the presence of tofacitinib in early RA mouse model

While the OT-II cells in our model act as the inflammation-inciting cells and potentiate the breakdown of self-tolerance, their specificity is irrelevant to the joint. On the other hand, the endogenous CD4$^+$ T cells were previously shown to be recruited into the joint in parallel with OT-II T cells and interact with DCs in a manner resembling cognate/antigen-specific interactions, thus potentially representing the autoreactive cells responding to the joint-specific epitopes.[468] Therefore, it was crucial to investigate whether the (pro-inflammatory) function of these endogenous CD4$^+$ T cells, directly contributing to/driving the autoimmune response (in our RA mouse model and potentially in the RA patients), could be affected by exposure to tofacitinib.

The endogenous CD4$^+$ CD45.1$^-$ T cell population was easily distinguishable and represented a comparable percentage of total lymph node cells between the treatment groups, particularly in the arthritic vehicle and drug-treated groups (Figure 5-3A). Endogenous CD4$^+$ T cells were further divided based on their CD44 expression levels, and a smaller CD44^{hi} population representing antigen-experienced cells was further analysed for either T-bet expression or IFNγ production capacity (Figure 5-4A). In contrast to OT-II cells, only a third of endogenous cells were of an activated CD44^{hi} phenotype. The percentage of CD44^{hi} T cells was significantly increased in the HAO-challenge mice compared to those receiving PBS injection, with the CD44^{hi} populations comparable in arthritic mice treated with vehicle and tofacitinib (Figure 5-4B). Since the HAO-immunized animals present with histological signs of arthritis, which in this model is initiated by collagen II-specific autoreactive CD4$^+$ T cell activation following HAO challenge[455], it is likely that a small number of those endogenous CD44^{hi} cells are newly primed CII-specific CD4$^+$ T cells. The rest of the CD44^{hi} cells might be recirculating memory CD4$^+$ T cells (likely) specific for other antigens originating from the joint tissues. Thus, if the newly primed CD4$^+$ T cells specific for collagen II or other joint-specific antigens, re-activated upon

HAO stimulation, would exhibit T-bet expression, this change might not be detectable within a whole CD45.1^{-} CD44^{hi} population. Thereby, the data would likely represent the alterations within the whole memory/antigen-experienced population of endogenous CD4^{+} T cells of joint-related specificity.

The endogenous CD44^{hi} T cells did not upregulate their T-bet expression upon the HAO challenge in vehicle-treated mice, and the drug treatment had no impact on the percentage of endogenous T-bet-positive cells (Figure 5-4C). The absence of detectable changes in T-bet levels might be due to the frequency of remaining endogenous CD4^{+} T-bet^{+} cells in the lymph node being too low, with the (CD4^{+}) T cells participating in the inflammatory response having migrated to the joint at this late time point (day 7) post-HAO challenge.

Despite the absence of T-bet upregulation, the endogenous CD44^{hi} T cells exhibited detectable IFNy production in all the treatment groups (Figure 5-4D), comparable to the lower levels of that in the transferred CD4^{+} T cells. However, similarly to the OT-II cells, endogenous CD44^{hi} T cells appear to retain their ability for IFNy production following tofacitinib exposure. Since a sizeable population of endogenous T cells is recruited to the inflamed joint at day 7 post-HAO challenge[468], where they could be directly re-activated either in antigen-specific or possibly bystander manner, it would be important to also assess joint endogenous CD4^{+} T cell function to complement and strengthen the current data.

Overall, the above results might suggest that the endogenous CD45.1^{-}CD4^{+}CD44^{hi} cells, recruited to the joint draining lymph node upon antigenic challenge and likely comprised of predominantly memory cells, appear non-responsive to tofacitinib treatment and maintain their IFNy production capacity upon re-stimulation.

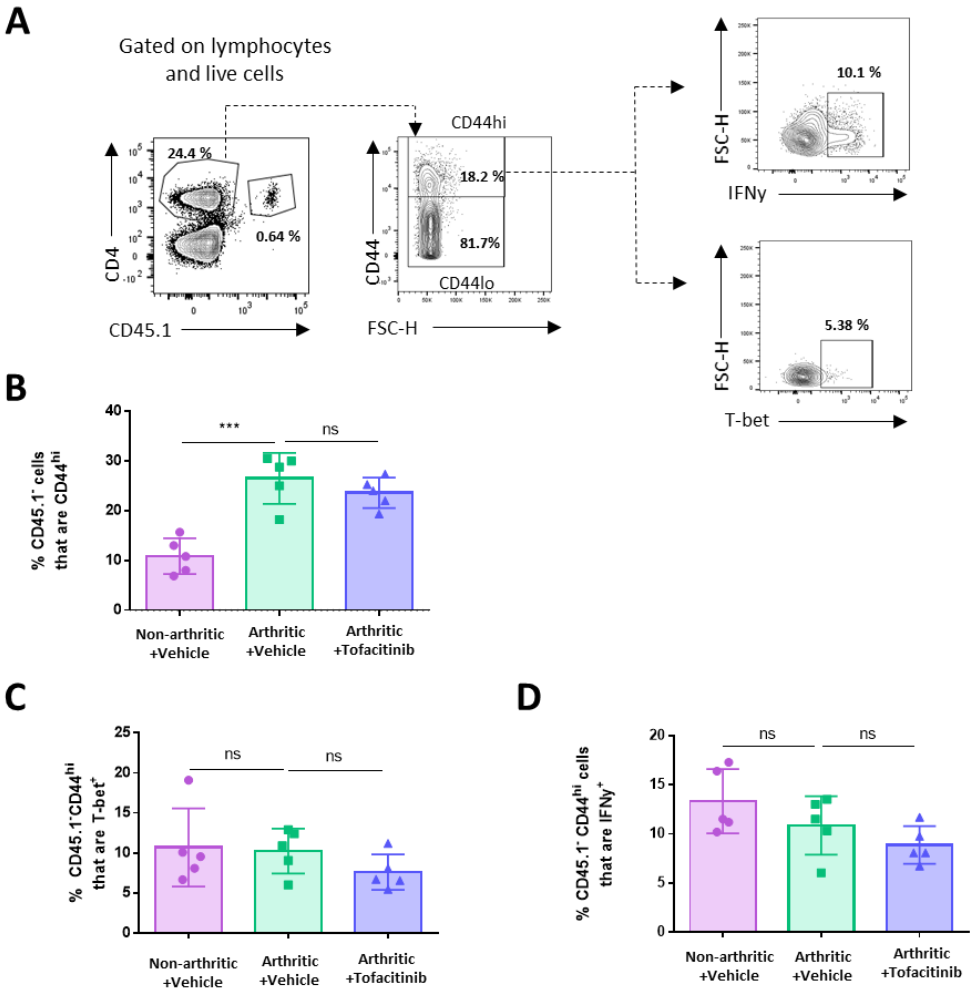


Figure 5-4. Endogenous CD4⁺ T cells exhibit no changes in T-bet expression or IFNy production in the presence of tofacitinib.

Experimental arthritis was induced in mice as described before (Fig.5-1). Starting one day before secondary challenge with HAO, mice received tofacitinib citrate treatment twice daily (25 mg/kg/) by oral gavage. Another HAO-challenged group and control (PBS) group received vehicle solution (containing 0.5% DMSO) twice daily by oral gavage. 7 days following HAO challenge mice were sacrificed and popliteal lymph nodes were collected for analysis by flow cytometry. Lymph nodes were mashed into single cell suspension and half of the cells from each lymph node were stimulated with PMA and Ionomycin with addition of Brefeldin A for 4 h at 37°C 5% CO_2, followed by intracellular staining for IFNy. Another half of the cells were stained for transcription factor T-bet. Both panels included surface staining for CD4, CD44 and CD45.1 surface markers. Lymphocytes were gated on live cells and adoptively transferred and endogenous cells were identified as $CD4^+CD45.1^+$ and $CD4^+CD45.1^-$ cell populations. Endogenous $CD4^+$ T cells were split into activated $CD44^{hi}$ and naïve $CD44^{lo}$ populations, and the $CD44^{hi}$ population was further gated on of T-bet positive or IFNy-producing cells (A). Percentage of endogenous $CD44^{hi}$ $CD4^+$ T cells was also analysed for each treatment group (B). Graphs depict percentage of $CD45.1^-$ $CD44^{hi}$ cells expressing T-bet (C) and capable of IFNy production (D). Graphs show mean ± SD, with n=5 per treatment group. The data represents results from a single experiment. Statistical differences between groups were assessed by One-way ANOVA and Turkey's multiple comparison test in GraphPad prism. **designates a *p* value of ≤ 0.01. ns=non-significant

5.2.3 Tofacitinib fails to affect the naïve endogenous CD4$^+$ T cell potential for T-bet expression and IFNy production.

In parallel with assessing the changes in endogenous CD4$^+$CD44^{hi} T cell population, I was interested to determine whether the exposure to tofacitinib *in vivo* might affect naive endogenous CD4$^+$ T cell subsequent activation and functional capacity. While limiting the autoreactive naïve T cell priming and effector/memory T cell re-activation are both highly desirable outcomes of the therapeutic intervention in RA, it would be equally important to preserve the naïve T cell population potential for activation and thus retain the ability to mount an efficient immune response towards infectious agents.

Endogenous CD4$^+$ T cells were identified as CD45.1$^-$ population in the popliteal lymph node, as previously, and further split based on their CD44 marker expression. Naïve cells were identified as having CD44^{lo} phenotype and either assessed for their T-bet expression or stimulated *ex vivo* with PMA/Ionomycin to determine their IFNy production potential (Figure 5-5A). Since the naïve CD44^{lo} cells were not stimulated prior to T-bet staining, the low proportion of T-bet-expressing cells was to be expected (Figure 5-5B). Upon *ex vivo* stimulation, a negligible proportion of CD44^{lo} T cells was found to be IFNy-positive in all treatment groups (Figure 5-5C), suggesting the failure of sufficient cell activation.

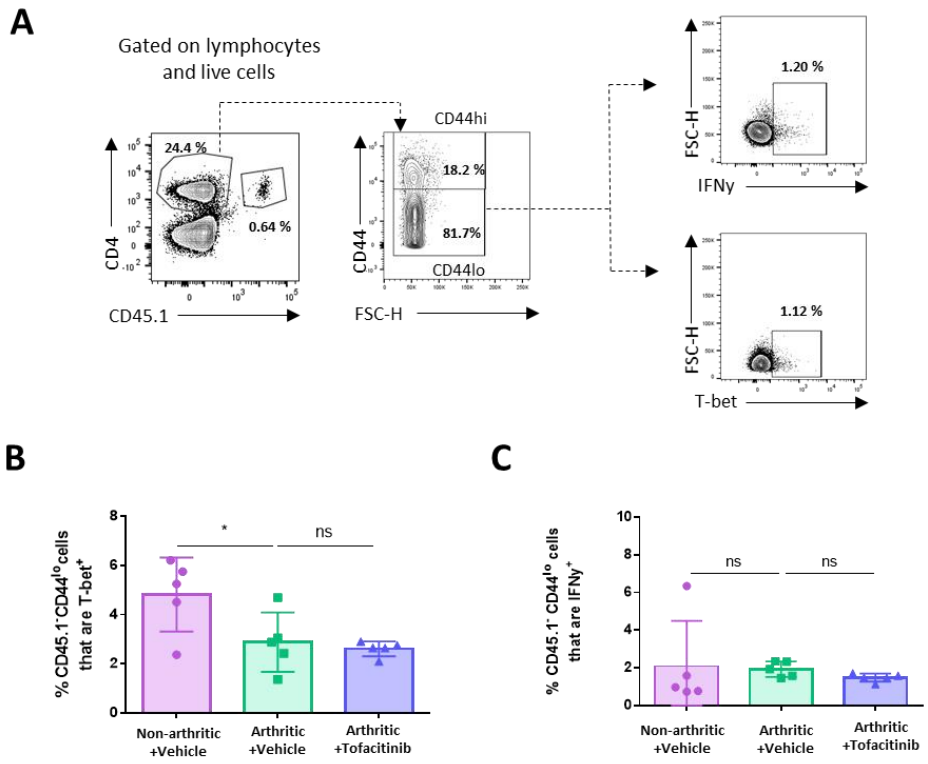


Figure 5-5. Tofacitinib fails to affect the capacity of naïve endogenous CD4⁺ T cells for T-bet expression and IFNy production.

Experimental arthritis was induced in mice as described before (Fig.5-1). Starting one day before a secondary challenge with HAO, mice received tofacitinib citrate treatment twice daily (25 mg/kg/) by oral gavage. Another HAO-challenged group and control (PBS) group received vehicle solution (containing 0.5% DMSO) twice daily by oral gavage. 7 days following HAO challenge mice were sacrificed and popliteal lymph nodes were collected for analysis by flow cytometry. Lymph nodes were mashed into single cell suspension and half of the cells from each lymph node were stimulated with PMA and Ionomycin with addition of Brefeldin A for 4 h at 37^{o}C 5% CO_2 , followed by intracellular staining for IFNy. Another half of the cells were stained for transcription factor T-bet. Both panels included surface staining for CD4, CD44, and CD45.1 surface markers. Lymphocytes were gated on live cells and adoptively transferred and endogenous cells were identified as $CD4^{+}CD45.1^{+}$ and $CD4^{+}CD45.1^{-}$ cell populations. Endogenous $CD4^{+}$ T cells were split into activated $CD44^{hi}$ and naïve $CD44^{lo}$ populations, and the $CD44^{lo}$ population was further gated on of T-bet positive or IFNy-producing cells (A). Graphs depict the percentage of $CD45.1^{-}$ $CD44^{lo}$ cells expressing T-bet (B) and capable of IFNy production (C). Graphs show mean ± SD, n=5 per treatment group. The data represents results from a single experiment. Statistical differences between groups were assessed by One-way ANOVA and Turkey's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05. ns=non-significant

5.2.4 Tofacitinib treatment has no impact on anti-OVA antibody response in RA mouse model.

Following the assessment of the tofacitinib treatment effect on the functional capacity of $CD4^+$ T cells, I sought to further investigate if the drug, potentially through interfering with T cell-dependent B cell activation, might affect the anti-OVA antibody responses. Serum samples were collected after animal sacrifice on day 7 post-HAO challenge and analysed by ELISA to determine the levels of anti-ovalbumin (OVA) antibodies. Both IFNy-dependent IgG2c (equivalent of IgG2a in C57BL/6 mice) and IL-4 dependent IgG1 isotypes of anti-OVA antibodies were tested to allow for the possibility of introducing bias from Th1 towards Th2 responses.

As expected, animals in all treatment groups were able to produce high levels of anti-OVA antibodies (Figure 5-6). The highest levels of both IgG1 (Figure 5-6A) and IgG2c (Figure 5-6B) antibodies were detected in arthritic (HAO-challenged) mice treated with vehicle, with PBS-challenged group exhibiting notably lower levels of both immunoglobulin isotypes. Animals receiving tofacitinib appeared to have the lowest levels of both IgG1 and IgG2c antibodies, which were significantly different for IgG1 when compared to those in the arthritic vehicle-treated group (Figure 5-6A). However, since the drug was introduced only 10 days following the first OVA administration, it would presumably be too late for it to affect naïve B cell responses, and the OVA-specific memory B cells would not yet be present in the system. Thus, although the difference introduced by tofacitinib treatment appears statistically significant at several serum dilutions, it is likely to have no biological relevance. Since the levels of IgG2c antibody exhibited a high variability between samples, it does not appear possible to distinguish any drug-induced changes from current data.

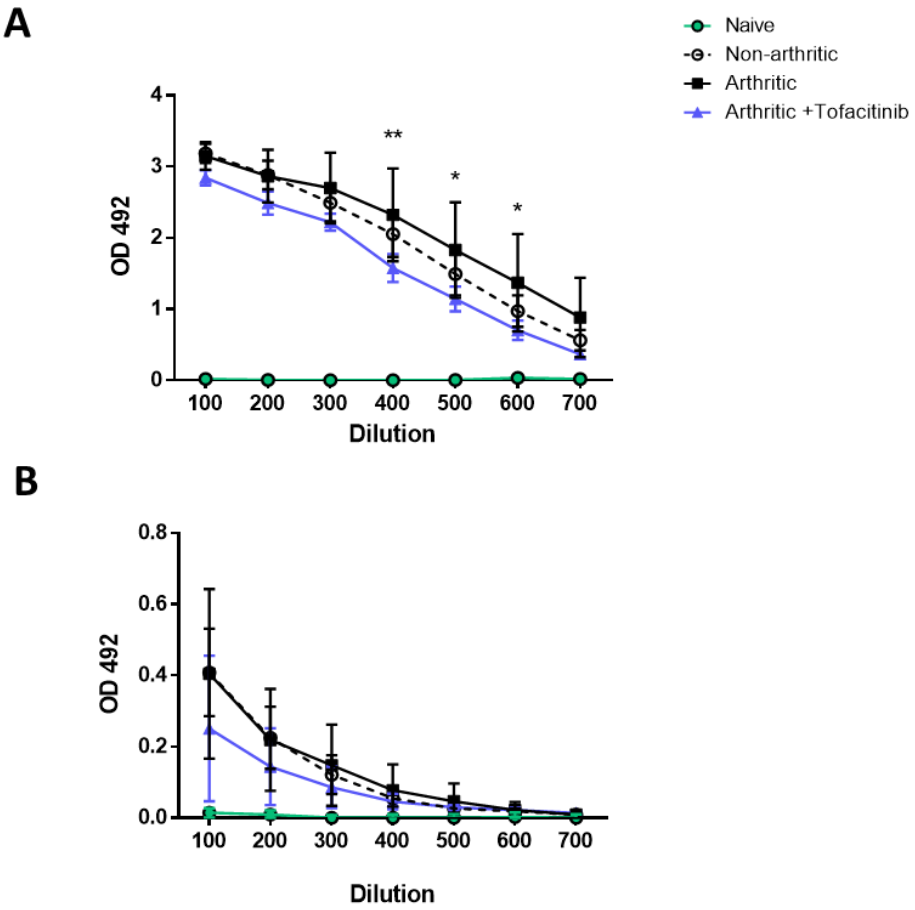


Figure 5-6. Tofacitinib administration has no impact on anti-OVA antibody responses in RA mouse model.

After animal sacrifice on day 7 post-HAO challenge, whole blood was collected by cardiac puncture and processed to obtain serum for assessment by ELISA. High-binding 96-well plates were coated overnight with chicken ovalbumin protein and serum from each sample was plated in duplicate at doubling dilutions. Serum from HAO-challenged ('arthritic') mice was used as a positive control. The negative control contained wild type ('naïve', green line) mouse serum. After 2 h incubation at RT, biotin - labelled IgG1 or IgG2c antibody were added for another 1 h incubation at RT. Following incubation, the Extravidin-Peroxidase substrate was added to each well and incubated for a further 30 min. SIGMA*FAST* OPD was used as a detection reagent, and the reaction was terminated using 10% sulphuric acid. Light absorbances were read using Sunrise Microplate reader at 492 nm. Graphs depict serum levels of anti-OVA IgG1 (A) and anti-OVA IgG2c (B) in each mouse group. Data shows mean ± SD (for each dilution) and represents 5 animals per group from a single experiment. Statistical differences between groups were assessed by performing two-way ANOVA and Turkey's multiple comparison test in GraphPad prism. The asterisks on the graph represent statistically significant differences between 'Arthritic' and 'Arthritic + Tofacitinib' groups. *designates a *p*6value of ≤ 0.05, ** ≤ 0.01.

5.2.5 Serum anti-collagen II antibody response in the RA mouse model.

Since the mouse model employed is known to develop arthritis following the loss of self-tolerance towards joint antigen collagen II, I sought the evidence of anti-collagen II (CII) antibody response similarly occurring in the recipient mice in my experiment. Serum samples were collected after animal sacrifice on day 7 post-HAO challenge and analysed by ELISA to determine the levels of total anti-CII IgG antibodies. The levels of total anti-CII IgG in all experimental groups appeared to be negligible in comparison to those in serum samples from collagen-induced arthritis (CIA) mice, used as positive controls (not shown), thus indicating the absence of anti-CII antibody response expected to develop in HAO-challenged mice at this specific time point (Figure 5-7). Since the IgG immunoglobulin levels are typically known to reach their peak between 2-3 weeks post-immunization during the primary antibody response, in the experiment repeat the model could be maintained for an additional week (post-challenge) to raise sufficient anti-CII antibody levels and to assess the potential effect of tofacitinib on the anti-CII humoral response.

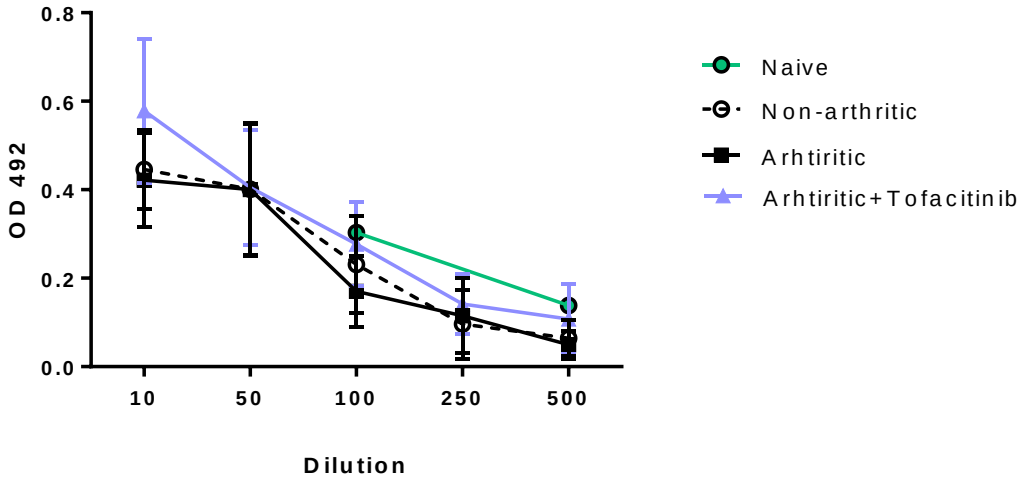


Figure 5-7. Serum anti-CII antibody titration in the early RA mouse model.

After animal sacrifice on day 7 post-HAO challenge, whole blood was collected by cardiac puncture and processed to obtain serum for assessment by ELISA. High-binding 96-well plates were coated overnight with chicken Type II Collagen (CII) and serum from each sample was plated in duplicate. Serum from the mouse with collagen-induced arthritis (CIA) was used as a positive control. The negative control contained wild type ('naïve', green line) mouse serum. After 2 h incubation at RT, HRP-labelled antibody for total IgG detection was added for subsequent 1 h incubation at RT. SIGMA*FAST* OPD was used for signal detection, and the reaction was terminated using 10% sulphuric acid. Light absorbances were read using Sunrise Microplate reader at 492 nm. The graph depicts serum levels of total anti-CII IgG for each treatment group. Each value is presented as a mean ± SD and represents 5 animals per group from a single experiment. The assay was repeated twice using the same serum samples with comparable results obtained between repeats.

5.2.6 Tofacitinib has no effect on paw inflammation but reduced inflammatory cell infiltrate and synovial hyperplasia in affected joints of arthritic mice.

Finally, I set out to investigate the effect of tofacitinib treatment on the development of acute experimental arthritis, characterized by paw inflammation and histological changes in the affected joints. Starting on day 1 after the HAO challenge, the development of arthritis was monitored by daily measurements of both left and right hind paw thickness until the experiment termination. Both HAO-challenged groups (vehicle and tofacitinib-treated animals) exhibited notable paw swelling, which was significantly greater than in the PBS-challenged group or days 1-3 and day 5 post-challenge, thus indicating a successful induction of arthritis (Figure 5-8A). The paw thickness remained largely unaltered in HAO/vehicle-treated arthritic mouse group throughout the course of experiment/disease, and the drug administration had no effect on the degree of paw thickness at any timepoint.

The hind limbs were removed and corresponding tissue sections from each limb, stained with H&E and toluidine blue, were histologically evaluated for the signs of pathology (inflammatory cell infiltration, synovial hyperplasia, articular cartilage degradation). As the HAO challenge was administered to the footpad, the joints of the tarsal bones proximal to the injection site and the surrounding tissue would be affected and thus were area of interest(Figure 5-8B). Joints of arthritic (HAO-challenged) mice receiving vehicle displayed extensive proinflammatory cell infiltration along the bottom side of the foot, paired with the development of hyperplastic synovium (2-3 cells thick)(Figure 5-8D). A mild but detectable loss of toluidine blue staining (in the area proximal to the inflamed synovium) indicated articular cartilage degradation also associated with pathology development in our model (Figure 5-8G). On observation, tofacitinib administration markedly reduced the extent of inflammatory cell infiltration, with infiltrates being more moderate in size and appearing as focal aggregates. The majority of drug-treated mice also displayed a normal, single-cell thick synovial membrane with characteristic lattice/honeycomb structure devoid of inflammatory cells (Figure 5-8E) and the articular cartilage integrity

was also preserved (Figure 5-8H). The loss of the above histological features in the tofacitinib-treated animals made their joints closely comparable to those of the healthy joints of the control (PBS-challenged) mice (Figure 5-8C,F). The blinded scoring of the sections for individual histological features confirmed both a significant increase in cell infiltration and the development of synovial hyperplasia in the arthritic vehicle-treated mice compared to control mouse (Figure 5-8J,K). The erosion of cartilage/bone score was also increased in the vehicle-treated group but was not substantial enough to reach statistical significance (Figure 5-8L). While the striking visual improvements in all three parameters were observed in tofacitinib-treated animals and the representative numerical scores appeared to be lower than in the vehicle-treated arthritic mouse group, these changes were not statistically significant (Figure 5-8J-K). Similarly, the average total histopathology score for all three parameters evaluated was significantly elevated in vehicle-treated arthritic mice relative to the control group, but the drug exposure did not result in statistically significant improvement of the score (Figure 5-8M).

Thereby, these results indicate that tofacitinib treatment following HAO challenge has no impact on the extent of hind paw inflammation. The drug treatment appears to reduce histopathological signs of the diseases, including inflammatory cell infiltration, synovial hyperplasia, and cartilage/bone degradation, but based on the numerical scoring these improvements are lacking significance.

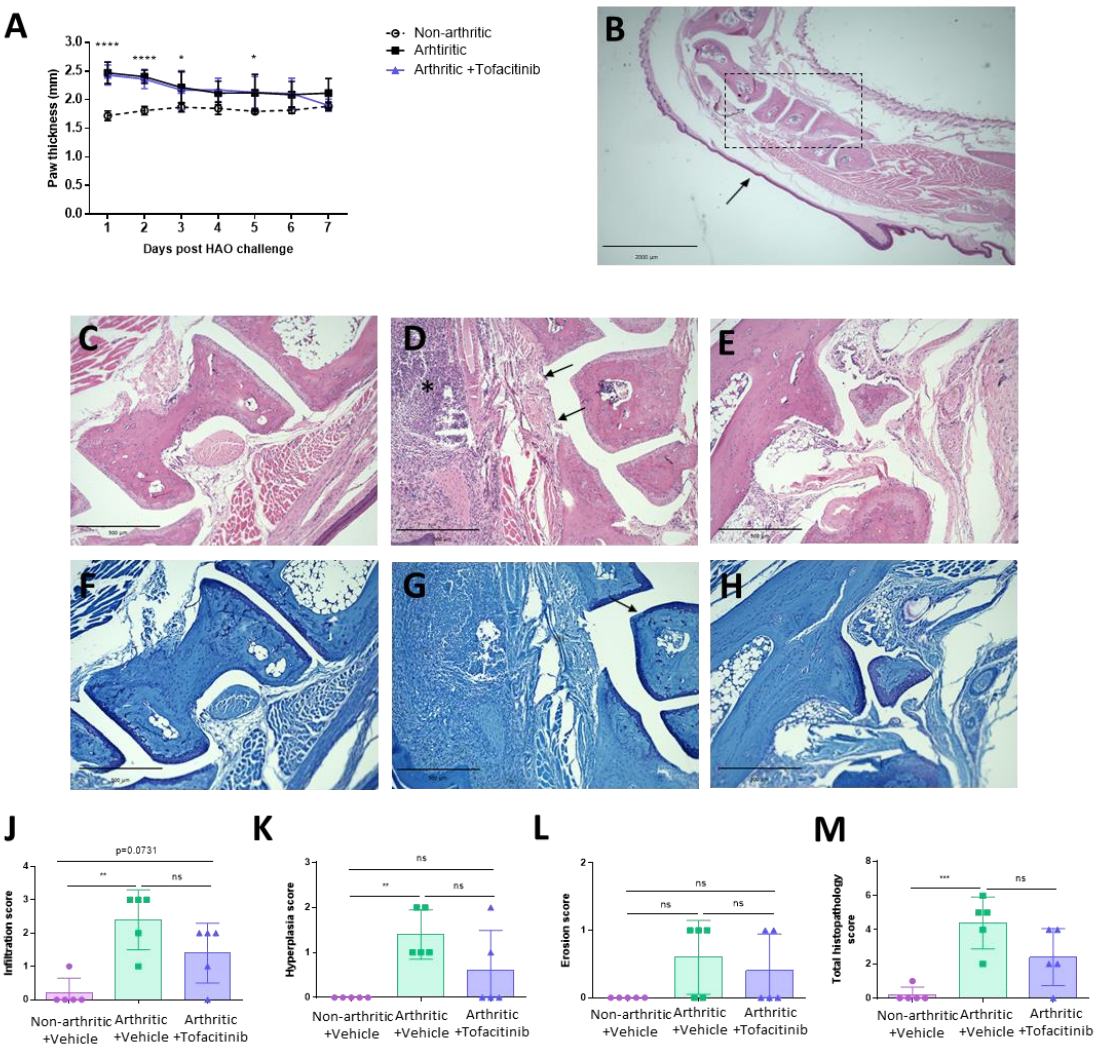


Figure 5-8. Tofacitinib treatment has no effect on hind paw swelling and not does ameliorate the inflammatory cell infiltrate and synovial hyperplasia in affected joints of arthritic mice.

(A) Changes in hind paw thickness of recipient mice from day 1 post-HAO challenge until the experiment termination. Results are depicted for the right paw, with similar measurements obtained for the left limb. Data represents mean ± SD , with n=5 for each treatment group. Statistical differences between groups were assessed by performing two-way ANOVA and Turkey's multiple comparison test in GraphPad prism. Hind limbs were collected after animal sacrifice on day 7 post-HAO challenge and sections stained with H&E (B-E) and toluidine blue (F-H). (B) Tarsal ankle joints proximal to the HAO injection site (arrow) were assessed for histopathological changes. Sections are representative of the joints of control mice challenged with PBS (C,F), HAO-challenged (arthritic) mice receiving vehicle treatment (D,G) and HAO-challenged (arthritic) mice treated with tofacitinib citrate. Arrows indicate synovial hyperplasia in H&E sections and cartilage erosion in toluidine blue-stained sections. An asterisk designates inflammatory cells infiltrates in H&E sections. All three histopathological features of early (acute) arthritis very most pronounced in arthritic vehicle-treated mice (D,G) with the control and tofacitinib-treated animals largely exhibiting no profound changes in the above parameters. Images were obtained using EVOS FL Auto 2 imaging system at original magnification x10. Each section was scored on the scale 0-3 for inflammatory cell infiltration (J), synovial hyperplasia (K) and cartilage and bone erosion (L), and the total score for above three parameters was calculated for each section (M). Data shows mean ± SD, with n=5 for each treatment group. The data represents a single experiment. Statistical differences between groups were assessed by performing one-way ANOVA and Turkey's multiple comparison test in GraphPad prism. *designates a *p*-value of ≤ 0.05, ** ≤ 0.01, **** ≤ 0.0001. ns = not significant.

5.3 Discussion

To investigate the impact of tofacitinib treatment on arthritis development, I have employed a mouse model of experimental early RA generated in our laboratory, which exhibited breakdown of self-tolerance following the transfer of Th1 $CD4^+$ T cells of joint-irrelevant specificity and subsequently developed joint antigen-specific humoral response alongside the histopathological characteristics similar to human disease.[455] The advantage of this model is the ability to therapeutically interfere at the pre-clinical disease stage/during self-tolerance breakdown and thereby assess the drug potential for suppressing self-tolerance breakdown through both effects on autoreactive (collagen-specific) $CD4^+$ T cells and the development of an autoantibody response. Since the pathology is promoted by trackable transgenic OT-II Th1 cells, the function of these cells alongside their endogenous counterparts can also be examined following tofacitinib exposure. Finally, the model enables to assess the potential for, and extent of, modulation of joint pathology by the drug to be addressed.

I have employed the drug in tofacitinib citrate formulation, which was designed for oral use in RA patients in the clinic and successfully employed earlier in the inflammation setting *in vivo* (Chapter 4). The drug dosage was also maintained at 25 mg/kg twice daily and for the purpose of this preliminary disease model experiment was administered by oral gavage. First, it was necessary to confirm the capacity of tofacitinib to inhibits its target signaling pathway in the acute RA mouse model setting. I was able to demonstrate that the drug administration dramatically suppressed circulating $CD4^+$ and $CD4^-$ leukocyte pSTAT3 phosphorylation levels at their basal state, thus confirming the effective inhibition by tofacitinib of its target JAK/STAT pathway in our animal model of early RA.

5.3.1 Tofacitinib does not affect adoptively transferred CD4$^+$ Th1 cell capacity for T-bet expression or IFNy production in the presence of tofacitinib in the early RA mouse model

At the time of rheumatoid arthritis diagnosis, which may be preceded by a pre-symptomatic/clinical disease development for up to a decade, both peripheral blood and synovium of patients are enriched with T cells of the antigen-experienced/memory phenotype[474,531] While these cells can be re-activated in an antigen-specific manner, they also are favourable candidates for antigen non-specific, bystander activation, which can occur in the cytokine-rich environment of inflamed synovium.[532] Thus, these activated T cells represent desirable therapeutic targets and should be investigated for their potential susceptibility to tofacitinib treatment. In Chapter 3 (Section 3.2.7) I demonstrated that antigen-specific CD4$^+$ T cells, primed in the absence of treatment and then re-challenged (with their cognate antigen) in the presence of tofacitinib *in vitro*, exhibited notably diminished T-bet expression but retained their IFNy production capacity. In the RA mouse model, the transgenic OVA$^{323-339}$-specific CD4$^+$ T cells, first polarized towards the Th1 subset *in vitro* and then receiving another cognate antigenic challenge *in vivo* prior to tofacitinib treatment initiation, represented the equivalent of antigen-experienced cells. Assessment of this adoptively transferred CD4$^+$ T cell population 7 days after another antigen-specific re-activation (with HAO) in the presence of the drug revealed low levels of T-bet expression but a normal capacity for IFNy production, which remained unaltered in the tofacitinib-treated animal group. A possible reason for a notably low T-bet expression in terminally differentiated Th1 cells could be the late time point (day 7) following antigenic challenge T-bet re-expression was previously shown to occur in the highest proportion of CD4$^+$ memory T cells at 72h following their re-activation, around the peak of the immune response.[518] Hence, the transcription factor expression is likely to be downregulated again by day 7, when the peak of the immune response has passed. The detected low levels of T-bet may also associate with the kinetics of the T cell response, with OT-II CD4$^+$ T cell recruitment to the inflamed joint occurring from day 1 post-challenge and culminating at day 4. Thereby, the OT-II cells remaining in the

popliteal lymph node at day 7 post-challenge may not actively participate in the ongoing inflammatory response.[468,455] Thereby, it would be of interest to examine the OT-II cells in the lymph nodes at earlier time points (days 2-3 post-challenge) Moreover, the OT-II cells were previously reported to interact with $CD11c^+$ dendritic cells in inflamed joints, implying antigen-specific reactivation directly in the affected tissue.[468] In this regard, assessment of the OT-II T cells from the affected (tarsal) joint tissues would provide additional insight into the tofacitinib potential to modulate their response to antigen-specific reactivation. So far, in this preliminary experiment and in line with my *in vitro* observations, I showed the adoptively transferred OT-II $CD4^+$ T cells to retain their ability for IFNy production following tofacitinib exposure in the context of experimental arthritis. My data to some extent is supported by the findings from the human study which subjected healthy volunteers to short-term (29 days) tofacitinib treatment and assessed their circulating $CD8^+$ T cells for IFNy production capacity. A modest reduction in the number of IFNy-secreting cells was observed half-way through the treatment in $CD8^+$ T cells, both activated in an antigen-specific manner or by the means of the anti-CD3/CD28 stimulation. However, the IFNy production in the above cell groups either normalized or returned to the baseline while still on tofacitinib treatment.[483]

5.3.2 Tofacitinib does not affect endogenous $CD4^+$ cell capacity for T-bet expression or IFNy production in the presence of tofacitinib in the early RA mouse model

Employing the adoptive transfer approach in generating an early RA model has also enabled me to distinguish the endogenous $CD45.1^-$ $CD4^+$ T cell population in the popliteal lymph nodes and attempt their functional potential assessment. The original study of the mouse model establishment demonstrated this endogenous population to contain some collagen II-specific (autoreactive) T cells, which are newly primed upon HAO challenge and presented with the self-antigen in the immunogenic fashion in the context of OT-II Th1 driven inflammation.[455] Also, a small fraction of the endogenous population might

represent previously activated OVA-specific $CD4^+$ T cells, which were primed during the initial OVA/CFA immunization and might have been reactivated upon secondary challenge. Finally, the majority of the endogenous $CD4^+$ T cells are likely to be memory cells of the joint and inciting antigen-irrelevant specificities, recruited to the draining lymph node in response to the ongoing inflammation.

When assessing the possible changes in T-bet expression and IFNy production, no notable differences were detected between the control (PBS-treated) and HAO-challenged groups, and the tofacitinib exposure in arthritic mice resulted in a very slight reduction in both marker levels which however was not statistically significant. One explanation for this observation could be drug-induced changes occurring only in a small fraction of cells (presumably collagen II-specific $CD4^+$ T cells), which could be hard to detect within a relatively large population of total endogenous $CD4^+$ T cells. Equally, it could also suggest that insufficient numbers of functional/activated endogenous $CD4^+$ T cells remain in the draining lymph node at the time of assessment. Thus, similarly to the OT-II cells, endogenous cells might be better examined early post-HAO challenge (days 1-2) while undergoing activation in the lymph node, prior to their maximal accumulation in the joint at day 4. Also, the examination of endogenous $CD4^+$ T cells recruited to the joint would allow their functional capacity and susceptibility to tofacitinib to be assessed.

While the endogenous $CD4^+$ T cells might represent a mixed population, it could be possible to specifically analyse the newly primed collagen II-specific population in the context of their response to the drug treatment. This could be achieved by stimulating the cells from the popliteal lymph node (and possibly joint) of each treatment group *ex vivo* with the collagen II-pulsed APCs and assessing the collagen II-specific $CD4^+$ T cell response by their levels of IFNy secretion, as done previously.[455] As demonstrated in Chapter 4 (Section 4.2.5), antigen-specific $CD4^+$ T cell priming *in vivo* is impaired in the presence of tofacitinib. Accordingly, one might predict that collagen II-specific $CD4^+$ T cells from the drug-treated animals would similarly demonstrate a diminished capacity for IFNy production upon priming.

The endogenous effector/memory $CD4^+$ T cells of irrelevant specificity represent another endogenous population of interest, particularly in the context of their non-specific re-activation in the inflamed joint microenvironment. Prendergast *et al*. demonstrated that adoptively transferred TEa TCR transgenic $CD4^+$ T cells (recognising $E\alpha^{52-68}$ peptide in the context of $I\text{-}A^b$) were recruited to the joints of arthritic mice even when their cognate antigen was not employed as the disease-inciting stimulus.[468] Extending this observation, another study, although in a slightly different context, proposed that the pre-activated OVA-specific OT-II cells, adoptively transferred into a tetanus toxin (TT) pre-immunised mouse containing TT-specific memory $CD4^+$ T cells, can also be re-activated upon TT secondary boost non-specifically by IL-2 and IFNy released from re-activated TT-specific $CD4^+$ T cells memory cells.[533] In line with this, Brennan *et al*. showed that the cytokine-activated T cells (Tck) can be generated by stimulating human peripheral blood cells with IL-2,IL-6, and TNFα, and confirmed that the $CD3^+CD4^+$CD45RO (resting) effector/memory cell subset closely phenotypically resembles RA synovial T cells and represent the most efficient functional effectors (cytokine-secreting) among Tck populations.[482] Overall, these studies suggest that previously activated/memory $CD4^+$ T cells may have a lower bystander activation threshold. Upon activation, bystander $CD4^+$ T cells have been shown to stimulate monocyte production of TNFα but not IL-10 *in vitro*, either through soluble factor secretion or cell-cell contact, contributing towards the imbalance of pro-and anti-inflammatory cytokines that would further exacerbate pathology[534]. Finally, with the majority of $CD4^+$ T cells in RA patient joint synovium exhibiting a memory phenotype[535], some demonstrating signs of partial activation[533], and thus representing likely candidates for bystander activation, the therapeutic intervention with JAK inhibitors might be beneficial for controlling this cell population. Due to the ability of tofacitinib to inhibit cytokine signaling, it might be of particular relevance for interfering with bystander cell activation. However, the drug efficiency in blocking human PBMC-derived T lymphocyte bystander activation and IFNy production has so far only been demonstrated *in vitro*. Our model could potentially be employed to further delineate the bystander memory $CD4^+$ T cell behaviour following tofacitinib treatment *in vivo* in the setting of the arthritic joint.

5.3.3 Tofacitinib administration has no impact on anti-OVA antibody response in RA mouse model.

While there is growing evidence that tofacitinib modulates $CD4^+$ T cell responses in both murine and human studies, the understanding of its impact on B cell function is very limited. The first notion of a link between JAK3 inhibition and B cell defects came from observations in patients with severe combined immunodeficiency (SCID) harbouring mutations impairing γc/JAK3 pathway signaling. While this did not affect B cell levels, intrinsic defects in cell proliferation, class switch-recombination and antibody production were seen.[536] Therefore, to evaluate the potential tofacitinib impact on B cell function in our early RA model, anti-OVA antibody responses were examined. Both anti-OVA IgG1 and IgG2c antibody production was detected in serum from all experimental mice, with the levels of IgG1 appearing significantly reduced at several concentrations in the serum of the drug-treated mice. However, as tofacitinib treatment was commenced 10 days after the first OVA protein challenge and thus antigen-specific B cell activation, it therefore would be unlikely to interfere with an initial induction of the anti-OVA antibody response. While there are no murine studies to date investigating the drug impact on B cell function, the above interpretation is supported by an investigation of human B cell responses. A study by Wang *et al*. convincingly demonstrated that tofacitinib exposure of naïve human $CD19^+$ B cells during their activation via the B cell receptor (BCR), CD40 ligand (CD40L) and cytokines (IL-4, IL-6 and IL-21) *in vitro* resulted in suppression of activation-induced cytidine deaminase (*AICDA*)and X-box binding protein 1(*XBP-1)*, B cell development-regulating genes, and abolished IgG production.[308] Similarly, when the drug was introduced 2 days following initial B cell activation, it was shown to not only abrogate fate-determining gene expression and thus impede cell differentiation and diminish immunoglobulin production, but also dramatically suppress pro-inflammatory IL-6 cytokine production while preserving regulatory IL-10 generation. In line with this, another study demonstrated impairment in naïve human B cell development into plasmablasts and associated immunoglobulin secretion upon activation in the presence of tofacitinib.[537] However, B cells already activated upon drug treatment retained normal *AICDA* expression and were capable of AICDA-

mediated class-switching.[537] Interestingly, only a moderate effect on total peripheral blood B cell function was achieved, potentially suggesting the functional capacity of circulating memory cells to be preserved despite tofacitinib administration. In the context of this evidence, I hereby propose that as the drug was introduced 10 days after B cell response initiation when the cells have presumably developed into antibody-secreting plasmablasts/short-lived plasma cells, the treatment was unlikely to induce prominent changes in anti-OVA B cell response. Thus, the statistically significant reduction in the anti-OVA IgG1 titres in drug-treated mice is likely to be of no biological relevance.

In addition, I have assessed the effect of tofacitinib on the autoantibody response to the main component of cartilage, type II collagen (CII), which indicates self-tolerance breakdown and is initiated following the induction of articular inflammation. However, the arthritic mice failed to develop a detectable anti-CII response and thus the differences in antibody titres between treatment groups could not be discriminated. Following the first encounter with the antigen, there is a latent phase lasting approximately 4-7 days during which naïve B cells are activated in an antigen-specific manner and subsequently undergo proliferation, receive T cell help and differentiate into antibody-secreting short-lived plasma cells[538], with the peak antibody levels reached by approximately 14 days following the challenge. Thus, extending our mouse model for another week and evaluating anti-CII antibody titres at 2 weeks post-HAO immunization might facilitate a better experimental read-out. As the drug treatment is initiated a day prior to HAO-challenge, I propose, based on the evidence from the previous human B cell *in vitro* studies[308,537], that tofacitinib would interfere with naïve B cell activation in response to collagen II and thereby notably diminish antibody class switching and anti-collagen II immunoglobulin IgG production. While it would not be possible to interfere during the breakdown of self-tolerance in RA patients, tofacitinib treatment could potentially limit the development of antibody responses towards neo-antigens generated or released during the course of the disease.

Since both IL-4 and IL-21, secreted by T follicular helper (Tfh) cells, are non-redundant in germinal centre B cell initiation and subsequent survival, maturation and effector response development[539], one of the ways tofacitinib

can exert an effect on B cells could be through direct inhibition of JAK1/JAK3-dependent IL-4 and IL-21 cytokine signaling. However, since differentiation of Tfh cells at early stages relies on IL-2 and IL-6 cytokines[540], also employing JAK/STAT pathway downstream of their receptors, potential interference with Tfh subset development could present another mechanism of tofacitinib interference with efficient humoral response generation. In addition, as tofacitinib was previously demonstrated, both in this thesis and in several other studies[305,516], to impair T cell polarization towards helper subtypes, this could also potentially limit Th cell help required for successful B cell activation by a low-valency antigen. Overall, a more in-depth understanding of the potential mechanisms through which tofacitinib modulates B cell responses and the subsequent outcomes is required, and our murine mouse model, exhibiting controlled and quantifiable autoantibody responses early in the disease might represent a useful tool for this purpose. Ultimately, the understanding of tofacitinib impact on B cell responses would inform on establishing the effective vaccination strategies in conjunction with tofacitinib treatment, so that the RA patients can successfully mount and preserve protective humoral immunity.

5.3.4 Tofacitinib treatment does not affect hind limb swelling, but reduced inflammatory cell infiltration and synovial hyperplasia in affected joints of arthritic mice

The mouse model employed also allowed examination of the tofacitinib ability to modulate affected paw inflammation and joint pathology. The drug administration throughout the course of the pathology did not appear to induce notable changes in the hind paw thickness. While tofacitinib effectively diminished inflammatory cell influx which contributes to oedema formation, it might not be able to control other aspects, such as vascular permeability. In contrast, in adjuvant-induced arthritis (AIA) rat model of established disease, a once-daily oral administration of considerably smaller drug dose resulted in significant paw volume reduction as soon as at 4 days post-treatment.[310] While this result cannot be directly correlated with my observations, it might

potentially suggest that tofacitinib is more effective at reducing inflammation in the established pathology but not during the acute disease phase.

Importantly, arthritic mice receiving tofacitinib treatment showed distinct reductions in inflammatory cell infiltration, suppression of synovial hyperplasia and amelioration of the articular cartilage damage. While these changes were striking and the joints from drug-treated mice exhibited tissue morphology closely comparable to that in the healthy control animals, the scoring results did not confirm the statistical significance of the tofacitinib-driven improvement at either of the key histological parameters. Variability in the vehicle control group and poor induction of disease in some mice might be the most likely explanations. This is further compounded by the relatively arbitrary scoring system employed, which only uses whole integers from 0 to 3 to evaluate each parameter and with only three parameters used, thus limiting the scoring precision and evaluation of the subtle changes occurring. Moreover, our RA mouse model is acute and recapitulates the early stages of the disease, thus the joint pathology is more subtle than that in the models of established RA and the possible drug-induced changes are more difficult to distinguish. Hence, while the numerical significance was not achieved in our experiment, the existing studies from a number of rodent models of established RA consistently report dramatic improvements in histopathological changes following tofacitinib administration. By using a more thorough scoring approach, Milici *et al.* demonstrated a dose-dependent reduction in the histological damage score in CIA mouse knee joints, with the maximal tofacitinib dose of 15 mg/kg/day achieving significant improvements when compared to vehicle-treated mice.[311] The histological observations, consistent with our findings, showed a profound reduction of inflammatory cell influx, synovial hypertrophy and limited cartilage destruction following drug treatment. Similar tofacitinib-induced improvements in the above histological features were described in another study employing CIA mouse model[305], as well as in rat AIA model[310] and mouse SKG model of spontaneous arthritis, with the treatment effect in the latter lasting for 3 weeks following drug withdrawal[312]. In addition, tofacitinib efficiently reduced the synovial invasion and the associated cartilage destruction in the synovium/cartilage tissue from RA patients, which was co-implanted into SCID mice receiving the drug treatment.[306] Thereby, although evaluating the tofacitinib impact in the

established disease context, the above studies convincingly demonstrate the drug ability to promote remarkable improvements in major histological features characteristic for arthritic joints, and thereby contextualise my findings as being biologically meaningful.

While the above animal studies consistently describe the tofacitinib-induced decrease in inflammatory cell infiltration and synovial hyperplasia, limited advances were made to investigate the (potential) mechanisms underlying these changes. As the cell recruitment to the joint is directed by chemokines that do not rely on JAK/STAT signaling pathway, the next best candidate to consider could be a pleiotropic/multifunctional cytokine highly expressed in RA and affecting a number of different immune cells related to disease pathogenesis. I thereby proposed that inhibition of IL-6 signaling by tofacitinib, which was demonstrated in this thesis and in other studies[305], might be one of the mechanisms limiting the inflammatory cell recruitment, development of hyperplastic synovium and the subsequent structural joint damage. IL-6 is produced mainly by resident synovial macrophages and fibroblast-like synoviocytes (FLSs) in the inflamed joints, and the infiltrating activated monocytes/macrophages also exhibit IL-6 production[541,542,543]. In FLSs, IL-6 can promote further activation and uncontrolled proliferation in the autocrine or paracrine fashion, thus contributing to the formation of the hyperplastic synovium[542,544]. IL-6 was also demonstrated to augment the production of monocyte chemotactic protein-1 (MCP-1, also known as CCL2) and IL-8, acting as chemokines, by both FLS and mononuclear cells, as well as induce the expression of adhesion molecule ICAM-1 on endothelial cell, thereby facilitating the recruitment of inflammatory cells to the joint.[545] In addition, IL-6 synergises with TNFα and IL-1ß in stimulating FLSs to secrete vascular growth endothelial factor (VEGF), which exhibits pro-angiogenic, pro-inflammatory and anti-apoptotic functions, while also contributing to osteoclastogenesis in the rheumatoid synovium.[546,547,548,549,550,551] Importantly, IL-6 can also promote cartilage degradation by directly inducing matrix metalloprotease (MMP) secretion by chondrocytes alongside with enhancing RANKL expression on osteoblasts and FLSs to promote osteoclastogenesis and subsequent bone resorption[552,553,554,555]. Thereby, through inhibiting IL-6 signaling in a number of inflammatory immune cells contributing to rheumatoid arthritis pathology

development, tofacitinib could potentially simultaneously modulate several inflammatory and destructive processes ongoing in the inflamed arthritic joint microenvironment.

6 General discussion

Despite the biological DMARDs successfully transforming the management of rheumatoid arthritis over the past two decades, there is a considerable unmet demand for the novel therapeutic modalities as the substantial proportion of patients fails to respond to the available treatments. As an alternative to large biological molecules targeting cytokines and their receptors extracellularly, the (orally available) small molecule compounds emerged which were designed to modulate intracellular signaling cascades through inhibiting associated protein kinases, and thereby regulate cell responses to the external stimuli. Among promising targets were Janus kinases (JAKs), tyrosine kinases employed by Type I and Type II cytokine receptors for transmitting the signals from a range of cytokines and hormone-like growth factors essential for immune and hematopoietic cell homeostasis, along with multiple pro-inflammatory cytokines implicated in the pathogenesis of several autoimmune conditions, including rheumatoid arthritis. The discovery of JAK3 genetic mutation resulting in the development of immunodeficient phenotype in humans provided the idea that the inhibitory effect of this JAK kinase targeting might be largely limited to the immune cell compartment, further emphasising the suitability of this molecular target for management of autoimmune disorders.[218,219] This line of research culminated in the development of tofacitinib, a potent selective JAK3/JAK1 inhibitor, which became the first-in-class compound approved for management of moderate-to-severe rheumatoid arthritis.[220] Tofacitinib demonstrated clinical efficacy non-inferior to biologic DMARDs and, remarkably, achieved clinical response in patients failing treatment with one or several biologic agents.[316,223,224] However, the immunomodulatory mechanisms underlying tofacitinib efficacy in the clinic only begin to be unravelled. To date, multiple studies have reported tofacitinib to notably diminish the ability of both murine and human $CD4^+$ T cells to proliferate, undergo differentiation, and produce pro-inflammatory cytokines,[305,306,312,484,485] but the specific stage of $CD4^+$ T cell activation (priming or re-challenge) at which the drug may exert its inhibitory effect was not yet established. This would allow to better understand the efficacy of the drug administration at different stages of RA in the context of its effect on $CD4^+$ T cell effector function and have potential implications in

reviewing the order of tofacitinib therapy utilization in the current disease management strategy.

6.1 Summary of the main findings

Chapter 3 examined the tofacitinib impact on $CD4^+$ T cell priming ***in vitro***. The key findings are:

- No impact on $CD4^+$ T cell activation and modest effect on proliferation
- Significant suppression of $CD4^+$ T cell differentiation into Th1 subset *in vitro*
- The persistence of above phenotype upon secondary challenge in the absence of tofacitinib
- No impact on the antigen-experienced $CD4^+$ T cell function *in vitro*

Chapter 4 investigated the tofacitinib effect on priming in the ***in vivo*** setting. The main findings are:

- Notable suppression of $CD4^+$ T cell polarization towards Th1 subset *in vivo*
- No impact on antigen-experienced (endogenous) $CD4^+$ T cell functional capacity *in vivo*

Chapter 5 explored the tofacitinib impact in the context of the **early acute RA mouse model**. The key results are:

- No effect on function of adoptively transferred Th1 $CD4^+$ T cells upon their re-activation

- No impact on endogenous antigen-experienced $CD4^+$ T cells functional capacity

- No conclusion available on drug impact on auto-antibody response generation

- Detectable (but non-significant) improvement of signs/features of joint pathology

My findings from **Chapter 3** showed that the presence of tofacitinib during antigen-specific $CD4^+$ T cell priming (by their cognate antigen-bearing DCs) *in vitro* did not affect $CD4^+$ T cell acquisition of activated phenotype, as evidenced by negligible changes in both early (CD69) and late (CD44, CD62L) activation marker expression, and appeared to modestly reduce cell proliferative potential, which was in line with diminished $CD4^+$ T cell ability to respond to proliferation-inducing IL-2 cytokine. However, tofacitinib profoundly reduced $CD4^+$ T cell differentiation towards Th1 lineage, as demonstrated by significantly inhibited transcription factor T-bet expression and the reduction of associated IFNy production. This effect (on both T-bet expression and cytokine production) was observed to persist upon $CD4^+$ T cell secondary re-activation in the absence of the drug. In contrast, CD4+ T cells primed in the absence of tofacitinib were shown to retain their capacity for IFNy production, despite diminished T-bet expression, when re-activated in the presence of JAK inhibitor.

The assessment of tofacitinib effects *in vivo* in **Chapter 4** appeared to replicate the results observed in the *in vitro* setting. Thus, I demonstrated herein that the adoptively transferred transgenic naïve (OT-II) $CD4^+$ T cells primed by their cognate peptide *in vivo* in the presence of tofacitinib failed to effectively polarize into Th1 subset, as evidenced by inhibited T-bet expression. However, the changes in subset-specific IFNy production were not significant, likely owing to delayed kinetics of the drug effect in the *in vivo* setting. On the other hand, tofacitinib treatment was found to suppress T-bet expression of endogenous antigen-experienced $CD4^+$ T cells but failed to affect their functional capacity, as demonstrated by sustained IFNy production upon *ex vivo* re-activation.

The results from **Chapter 5** demonstrated that tofacitinib fails to impact on IFNγ production of adoptively transferred Th1 $CD4^+$ T cells upon their re-activation in the murine model of early RA. Similarly, the endogenous antigen-experienced $CD4^+$ T cells in the same model preserved their T-bet expression and IFNγ production capacity following drug exposure. Thus, the impact of tofacitinib treatment on antigen-experienced $CD4^+$ T cells in this RA model is consistent with those observed both *in vitro* and in *vivo* settings (in Chapters 3 and 4, respectively). I was not able to assess the tofacitinib effect on autoantibody response generation/self-tolerance breakdown, characteristic for our early RA mouse model, due to arthritic animals failing to raise sufficient levels of (anti-collagen II) antibodies. Lastly, I have observed marked reduction in inflammatory infiltrates and synovial hyperplasia in the joints of tofacitinib-treated mice, however, these changes did not appear significant likely due to mild degree of pathology and scoring system limitations.

6.2 Clinical implications

The identification of tofacitinib ability to interfere with effective $CD4^+$ T cell differentiation upon priming in the context of cognate $CD4^+$ T cell-DC interaction, presented in this thesis, may potentially have valuable implication in the clinical setting.

Thus, tofacitinib treatment could be effective during the initial event of autoreactive $CD4^+$ T cell priming by self-epitope-bearing mature DC, which is thought to trigger self-tolerance breakdown and initiation of the autoimmune response in RA (Figure 6-1). However, while many genetic and environmental risk factors were identified that would predispose the individual for loss of self-tolerance, currently there is no way to either predict this event to occur with certainty or to identify the specific time when it might ensue. Therefore, utilization of tofacitinib at this stage of RA pathology might not be as yet feasible clinically.

Upon the initial self-tolerance breakdown, the autoimmune response is further perpetuated by continuous priming of $CD4^+$ T cells of new antigenic specificities by newly formed epitopes or epitopes released following inflammation-driven tissue damage, a process known as epitope spreading. Epitope spreading was reported to predominantly occur during the stage of pre-clinical inflammation and was suggested to play a critical role in the transition from pre-clinical to clinically manifested RA.[556,557] Consequently, tofacitinib treatment at the pre-clinical stage of the disease might interfere with the cycle of 'new' naive T cells continuously breaching self-tolerance, thereby preventing them feeding forward into ongoing inflammatory response and possibly ultimately impeding the disease progression towards clinical disease (Figure 6-1).

Some evidence suggests that epitope spreading might also continue after clinical onset of disease. Thus, a study comparing dominance of T cell clones in recent onset and established RA patient synovium demonstrated that the number of highly expanded clones is significantly lower in established RA, proposing this might result from these clones losing their initial dominance due to additional autoreactive T cell clones being newly activated during the ongoing inflammatory response.[558] In line with that, another study reported the T cells from shared epitope-bearing patients with recent onset RA to respond only to citrullinated aggrecan or no epitope, while patients with established disease showed response to more than one citrullinated self-epitope, with possible underlying mechanisms suggested to be epitope spreading continuing throughout development of symptomatic/clinically manifested RA.[559] Following from the above, it could be proposed that therapeutic interference with tofacitinib would also be effective early in clinical onset RA, as it could potentially limit the evolution of $CD4^+$ T cell response and thereby halt the progression towards established disease (Figure 6-1).

However, the therapeutic efficacy of tofacitinib cannot be solely explained by modulation of $CD4^+$ T cell responses upon antigen-specific priming. The visible improvement in joint pathology in the absence of tofacitinib effect on the function of either of $CD4^+$ T cell populations in our early RA mouse model suggests that tofacitinib might also have an impact on other inflammatory cells contributing to the pathology development. The remarkably rapid mode of

clinically meaningful improvements in the established disease in CIA mouse model and in the patients with active RA refractory to methotrexate, reported previously, indicate that tofacitinib might exert its immediate therapeutic effect by directly interfering with the cytokine-dependent activity of innate immune and/or synovial resident cells.[305,560] Substantiating this notion, the tofacitinib treatment of fibroblast-like synoviocytes(from RA patients was shown to supress the TNF-mediated production of chemokines promoting T lymphocyte and monocyte recruitment (IP-10 and MCP-1, respectively).[561] Furthermore, tofacitinib was also reported to diminish the expression of T cell chemokines along with (pro-inflammatory) cytokine IL-6 in RA synovial macrophages. Importantly, the amelioration of arthritis in K/BxN mice by tofacitinib further supported the idea that the clinical drug efficacy partly relies on innate immune cell function modulation, as arthritis in this particular model is mediated exclusively by innate immune cells.[562] Collectively, the current evidence suggests that tofacitinib might in part drive the improvement in RA pathology through directly suppressing pathogenic function of macrophages and synovial fibroblasts and in turn ameliorating recruitment of adaptive immune cells to the joint. Notably, tofacitinib was also proposed to indirectly inhibit macrophage and synovial fibroblast cytokine production as well as osteoclast differentiation and bone-resorption capacity by suppressing T cell differentiation and RANKL production, respectively.[306,310] Thus, the clinical efficacy of tofacitinib could be attributed to its unique mode of action, which allows to concurrently modulate innate and adaptive immune cell function as well as potentially interfere with their cytokine-mediated cross-talk, thereby breaking the ‘vicious’ cycle of synovial inflammation.

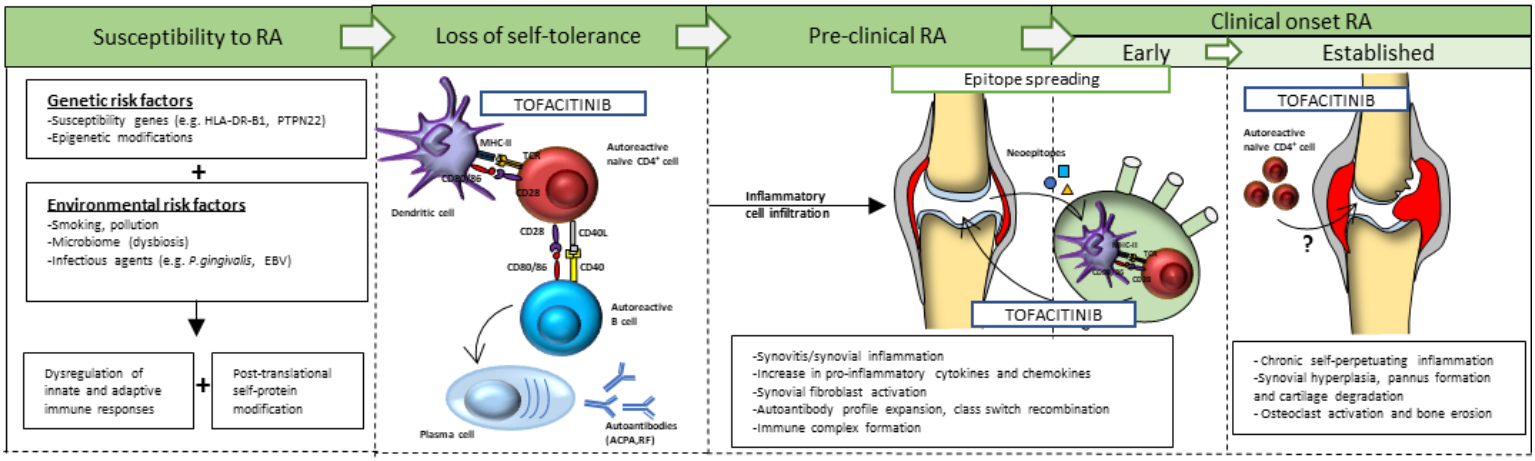


Figure 6-1. The course of rheumatoid arthritis development and the stages during which tofacitinib might have an impact on DC-mediated CD4⁺ T cell priming.
Based on my findings, tofacitinib might be able to interfere with DC-CD4⁺ T cell interactions at several stages of disease development. Tofacitinib treatment during the 'breach of self-tolerance' event would prevent the autoreactive CD4⁺ T cell priming by self-antigen bearing (mature) DC, and thereby would prevent the initiation of the autoimmune response, including activation of autoreactive B cells and subsequent autoantibody production. The JAK inhibitor might also be effective during pre-clinical and possibly early clinical/symptomatic RA, as it could interrupt the continuous priming of CD4⁺ T cells (in joint-draining lymph nodes) in response to neoepitopes released from the inflamed joint tissue. In turn, this would preclude the migration of newly activated/primed CD4⁺ T cells to the joint and them potentiating further damage and release of other neoepitopes, as well as interfere with 'help' from these CD4⁺ T cells to B cells in the generation of pathogenic autoantibodies of new specificities, thereby preventing autoantibody-mediated inflammatory and joint resident cell activation and associated joint damage. Clinically, tofacitinib interference during epitope spreading might, therefore, attenuate the disease progression. Additionally, if the 'new' naïve T cells continue to be recruited to the joint and contribute to the pathology during the chronic inflammation, tofacitinib could also be effective in interfering with their activation, and might ,therefore, be utilized at the established phase of RA.

6.3 Future experiments

The murine models utilized for this project provide useful platforms for further assessment of tofacitinib impact on DC-CD4⁺ T cell interactions *in vivo*. One of the options would be to elucidate the effect of tofacitinib treatment on *in vivo* CD4⁺ T cell differentiation into other T helper subsets. Since in this project, in line with observations from another murine RA model study,[312] tofacitinib administered during CD4⁺ T cell priming did not diminish the levels of IL-10 in the co-culture supernatant, it could be of interest to further investigate whether the drug impacts naïve T cell polarization into IL-10-secreting Tregs. By employing our adoptive transfer model system (Chapter 4), the effect of tofacitinib presence on naïve CD4⁺ T cell differentiation into Tregs could be

assessed in the *in vivo* setting. Given the drug has no impact on Treg polarization and functional capacity, this might provide an additional mechanism underlying tofacitinib efficacy in RA.

While extensive research is focused on tofacitinib ability to modulate $CD4^+$ T cell responses, less is known about the JAK inhibitor impact on dendritic cell function. Both human monocyte-derived and murine bone-marrow derived DCs treated with tofacitinib during their maturation *in vitro* were shown to develop a 'tolerogenic' phenotype and exhibit impaired ability to provide co-stimulatory and cytokine signals crucial for efficient $CD4^+$ T cell priming.[309,514] Since in our adoptive transfer model tofacitinib treatment is initiated prior to administration of stimulus inducing endogenous DC maturation, it provides an excellent setting for assessment of alterations in DC functional potential in response to tofacitinib *in* vivo, which has not been done previously.

It would be of particular interest to test the suggestion that tofacitinib might limit the diversity of $CD4^+$ T cell repertoire by inhibiting the continuous priming of these cells throughout the disease development. A 'breach of tolerance' mouse model of RA employed in this project, was recently adapted in our lab to study the changes in TCR repertoire diversity of endogenous $CD4^+$ T cells (in joints and popliteal lymph nodes) at specific timepoints following primary (HAO) footpad challenge (early timepoint) and secondary challenge (late timepoint). Interestingly, the clonal $CD4^+$ T cell diversity in popliteal lymph nodes at late timepoint increased compared to that at early timepoint, suggesting a potential epitope spreading occurring in this model. Thus, administration of tofacitinib in this model prior to/beginning at the early time point and assessement of changes in the endogenous $CD4^+$ T cell clonality at the late timepoint could be a feasible way to test the above proposal. In ideal scenario, the comparison of TCR repertoire diversity in patients who commenced tofacitinib treatment close to RA onset and those who initiated treatment later in the disease might potentially inform on the drug capacity to limit epitope spreading in clinical setting, and might subsequently have implications in reviewing the time in the disease course when the compound is administered.

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