Dissertation

Th1 cells in human diseases

Submitted by

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1. Statutory Declaration

"Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice".

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2. Disclosures

Part of this thesis (the COVID-19 data) was originally published in The Journal of Immunology. Rupp J[1], Dreo B[1], Gütl K[2], Fessler J[1], Moser A[3], Haditsch B[3], Schilcher G [4], Matzkies LM[5], Steinmetz I[5], Greinix H[6], Stradner MH[1]. 2021. T Cell Phenotyping in Individuals Hospitalized with COVID-19. J Immunol. Vol:206. Issue7. Copyright © 2021 by The American Association of Immunologists, Inc.

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All co-authors have agreed to use our published data in the dissertation and permission of the Journal of Immunology, to include the published data in my dissertation, has been obtained.

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4. Table of Content

5. Abbreviations and Definitions

COVID-19	coronavirus disease 2019
SARS-CoV-2	Severe Acute Respiratory Syndrome Corona Virus-2
RA	rheumatoid arthritis
Th1	T-helper 1
Th2	T-helper 2
Th17	T-helper 17
Treg	regulatory T cell
TFH	T follicular helper cell
TCR	T cell receptor
MHC	major histocompatibility complex
HLA	human leucocyte antigen
HLA-DR4	HLA-DRB1*0401
IFN-γ	interferon-γ
IL	interleukin
TNF	tumor necrosis factor
STAT	signal transducer and activator of transcription
ARDS	acute respiratory distress syndrome
CRS	cytokine release synd
PD-1	programmed death protein-1
Tim3	and mucin domain-containing protein3
GM-CSF	granulocyte macrophage colony stimulating factor
IP-10	interferon-inducible protein 10
MCP1	chemotactic protein 1
ACPA	anti-citrullinated protein antibodies
CIA	collagen-induced arthritis model
PBMC	peripheral blood mononuclear cells
NSG-DR4	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} *H2-Ab1*^{tm1Doi} Tg(HLA-DRB1)31Dmz/SzJ
NK	natural killer cell
IL2rg	the IL-2 receptor γ-chain locus
iv	intravenous
ip	intaperitoneal
sc	subcutaneous
ih	intrahepatic
GvHD	graft versus host disease
HC	healthy control
Ct	cycle threshold

6. Abstract in German

T-Helfer 1 (Th1)-Zellen sind an verschiedenen Krankheiten beteiligt. In dieser Arbeit haben wir uns auf eine Infektionskrankheit (Coronavirus-Krankheit 2019, COVID-19) und eine Autoimmunerkrankung (rheumatoide Arthritis, RA) konzentriert und zwei Fragestellungen behandelt: I) Identifizierung von T-Zell-Reaktionen bei Patienten mit akuter COVID-19 und II) Verbesserung des Verständnisses der Arthritisentstehung, der Bedeutung von Th1-Zellen und der Identifizierung der arthritogenen Immunzellen unter Verwendung eines neuen humanisierten Mausmodells der RA. Th1 gehören zusammen mit $CD8^+$ T-Zellen zu den antiviralen Immunzellen. Darüber hinaus hat sich gezeigt, dass eine ausgeprägte Th1-Antwort zur Virusabwehr führt und mit einer guten Prognose verbunden ist. Wir konnten signifikante Veränderungen in der T-Zell-Verteilung, aber auch in der Aktivierung und Proliferation von COVID-19 Patient*innen im Vergleich zu gesunden Spender*innen feststellen. Diese Veränderungen könnten durch den IL-6-Spiegel im Plasma moduliert werden. Bei RA gehören Th1 zusammen mit Th17 und regulatorischen T-Zellen (Tregs) zu den wichtigsten Immunzellen. Die genaue Pathogenese für die Entwicklung dieser unheilbaren und schmerzhaften Krankheit und die Art und Weise, wie arthritogene T-Zellen sie auslösen, ist jedoch immer noch nicht klar. HLA-Klasse-II-Gene wie HLA-DRB1*0401 (HLA-DR4) weisen jedoch das größte genetische Risiko auf und lassen auf eine Beteiligung von $CD4^+$ T-Zellen schließen. Bestehende Mausmodelle ahmen zwar bestimmte Aspekte der Krankheit nach, können aber das menschliche Immunsystem nicht vollständig nachbilden. Unser Ziel war es, arthritogene Zellen zu identifizieren, indem wir HLA-$DR4^+$ periphere mononukleäre Blutzellen (PBMC) von RA-Patienten auf immundefiziente NSG-DR4-Mäuse übertragen. Dadurch konnten wir ein neuartiges Mausmodell mit entzündlicher Gelenkerkrankung generieren, dass nur durch den Transfer menschlicher Immunzellen ausgelöst wird. Die Arthritisentwicklung wurde durch mikrocomputertomographische und histologishe Untersuchung der Gelenke überwacht. Hier zeigen wir, dass $DR4^+$ T-Zellen aus dem peripheren Blut von RA-Patienten in der Lage sind, eine RA-ähnliche Erkrankung in NSG-DR4-Mäusen auszulösen. Th1-Zellen dominierten die Zusammensetzung der menschlichen Immunzellen im Mausmodel, während die Tregs im Vergleich zur Zusammensetzung der Spender-PBMC vermindert waren. Der Transfer von in vitro Th1-polarisierten T-Zellen erhöhte die Arthritisinzidenz. Auf der Grundlage unserer Daten vermuten wir, dass arthritogene Zellen, die im peripheren Blut von RA-Patienten zu finden sind, in der Lage sind, eine RA-ähnliche Erkrankung in NSG-DR4-Mäusen auszulösen.

7. Abstract in English

T-helper 1 (Th1) cells play a major role in human health and are implicated in different diseases. Here we focused on an infectious disease (coronavirus disease 2019, COVID-19) and an autoimmune disease (rheumatoid arthritis, RA). The approach of this study was to: I) identify T cell responses, in patients with acute COVID-19 and II) improve our understanding of how arthritis is initiated, the implication of Th1 cells, and to identify the arthritogenic immune cells by using a novel humanized mouse model of RA. Th1 belong together with $CD8^+$ T cell to the antiviral subsets. Furthermore, it has been shown that prominent Th1 responses lead to viral clearance and are linked to good prognosis. Using multicolor flow cytometry, we profiled the T cell composition including markers for activation and proliferation. Comparing hospitalized COVID-19 patients to healthy controls, we see significant changes in the T cell distribution but also activation and proliferation. These alterations might be modulated by IL-6 levels in the plasma, which may impair viral clearance by blunting the antiviral T cell responses. In RA Th1 belong together with Th17 and regulatory T cells (Tregs) to the key subsets. However, the exact pathogenic pathway for developing this incurable and painful disease and how arthritogenic T cells trigger it is still elusive. HLA class II genes however, such as HLA-DRB1*0401 (HLA-DR4), confer the strongest genetic risk and suggest involvement of $CD4^+$ T cells. Existing mouse models mimic specific aspects of the disease but do not fully recapitulate the human immune system. Thereby current research is limited and would profit from a humanized mouse (hu-mice) model. We aimed to identify arthritogenic cells by transferring HLA-$DR4^+$ peripheral blood mononuclear cells (PBMC) of RA patients to immunodeficient NSG-DR4 mice. Thereby generating a novel mouse model with inflammatory joint disease, only triggered by the transfer of human immune cells. Human engraftment assessed using multicolor flow cytometry. Development of RA was monitored by examination of the joints, followed by micro computed tomography analysis and histology. Here, we show that $DR4^+$ T cells of the peripheral blood of RA patients are capable of inducing an RA-like disease in NSG-DR4 mice. These mice recapitulate different hallmarks of the disease including immune cell infiltration, pannus formation, increased osteoclastogenesis, cartilage damage, and bone erosions. Th1 cells, dominated the human immune cell composition in hu-mice, while Tregs were diminished compared to donor PBMC composition. Based on our data we suggest that arthritogenic cells, found in the peripheral blood of RA patients, are capable of inducing an RA-like disease in NSG-DR4 mice.

8. Introduction

8.1. T cells in a healthy immune system and characterization of Th1 cells

Human health is strongly regulated by the immune system, which consists of the innate and adaptive immune system. This work fill focus on the adaptive immune system, more precisely on T Lymphocytes and how dysregulation affect human diseases resulting in for example, infectious or autoimmune diseases.

$CD4^+$ and $CD8^+$ T cells play central roles in the regulation and function of the immune system and develop during thymic maturation. Progenitor T cells, which express CD34 enter the thymus and undergo T cell receptor (TCR) α and β gene rearrangement (1–4). Rearrangement of the TCRβ locus lead to $CD4^+CD8^+$ double positive thymocytes, followed by TCRα rearrangements. αβT cells interact with self-peptides presented by major histocompatibility complex (MHC) resulting in either $CD4^+$ or $CD8^+$ single positive T cells (depending on MHC class II or I presentation; positive selection). Failure in any of these steps will lead to cell death by apoptosis. Negative selection leads to TCR mediated apoptosis and is a critical mechanism to delete self-reactive thymocytes (2–4). Thereby, self-tolerant T cells are generated. In autoimmune diseases autoreactive T cells escape negative selection leading to mature self-reactive T cells in the periphery that trigger autoimmunity (5). After the selection process mature naïve $CD4^+CD45RA^+CCR7^+$ or $CD8^+$ $CD45RA^+CCR7^+$ T cells migrate to lymphoid organs, where they encounter antigens leading to activation, extensive proliferation, and differentiation into effector cells that mediate infection clearance (4).

Peripheral T cells comprise of different subsets including naïve, memory, and regulatory T cells (Tregs). Memory T cells dominate the circulating T cells and are subdivided into central memory ($CD45RA^-CCR7^+$) and effector memory ($CD45RA^-$ $CCR7^-$) (6). Both, central and effector memory, can produce IL-2 and other effector cytokines but differ in their proliferative capacity and homing profiles (6,7). The major $CD4^+$ T helper (Th) effector cells include: Th1, Th2, Th17, regulatory T cell (Treg), and follicular T helper (TFH) cells (Figure 1) (8–11). $CD4^+$ T helper cell

characterization is based on the expression of surface marker and unique transcription factors and cytokine production.

Th1 and Th2 were discovered first and secrete different signature cytokines. Th1 belong to the antiviral and antibacterial immune cells and produce pro-inflammatory interferon-γ (IFN-γ), interleukin (IL) -2, and tumor necrosis factor (TNF) –α. Furthermore, it has been shown that presence of IL-12 leads to polarization towards Th1 cells (12) and activates the signal transducer and activator of transcription (Stat)3 and Stat4 (13). Th2 cells are important against extracellular pathogens and express IL-4, IL-5, and IL-13 (9). T-bet and GATA3 are the master transcription factors for Th1 and Th2, respectively (14). Th17 cells are implicated in anti-fungal and bacterial responses (15) and characterized by IL17 production (11,16) and RORγt expression (17). Tregs produce immunosuppressive IL-10 and can thereby limit the inflammatory response of Th1 and Th17 cells (18). Each effector subset differentiates from naïve T cells upon different cytokine stimulation. IL-12 and IL-4 are the master cytokines to promote Th1 and Th2 polarization, respectively (19). TGF-β, IL-6, and IL-21 promote Th17 differentiation (20,21). TGF-β has a dual role as it can also induce, together with IL-2, Treg differentiation (20,22). IL-6 and IL-21 can regulate TFH differentiation (23) (Figure 1).

In healthy adults, ranging from 20 to 65 years, IFN-γ producing $CD4^+$ T cells range from 15.31% to 34.98% (mean 24.09%). When compared to patients with an autoimmune disease, more precisely patients with systemic lupus erythematosus, increased numbers were observed (mean 34.98%) (24). In another study that included 150 healthy donors (20-70 years of age), Th1 ($CD4^+IFN\text{-}\gamma^+$) reference values range from 23.78% to 51.07% (25).

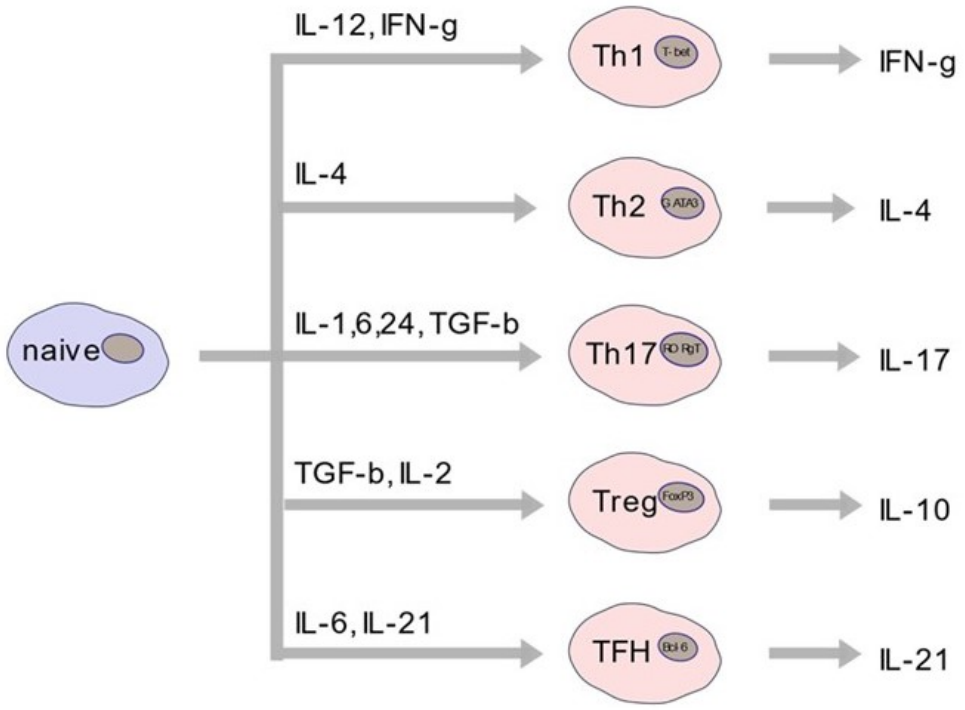

Figure 1 Overview of CD4$^+$ T cell subsets. A simplified model that summarizes master cytokines and transcription factors to generate different human CD4$^+$ subsets. Each subset secrets specific cytokines

8.2. COVID-19

8.2.1. COVID-19 – transmission, clinical features, and diagnosis

The coronavirus disease 2019 (COVID-19) 19 is a zoonotic infectious disease caused by the novel coronavirus defined as Severe Acute Respiratory Syndrome Corona Virus-2 (SARS-CoV-2). Due to the rapid international spreading, it has been declared as a pandemic (26). Transmission happens mainly via aerosols (27) and symptoms range from asymptomatic to mild and severe illness, including dry cough, dyspnea, fever, and pneumonia, which can be fatal (28). There are six other human coronaviruses known, four that cause minor symptoms (HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1) and two that lead to a severe disease (SARS-CoV and MERS-CoV) (29). Comparing the novel SARS-CoV-2 to the later, there are some similarities including transmission, increased disease severity with age, clinical symptoms, genetics (79.6% genome sequence similarity with SARS-CoV), lymphopenia, and a cytokine release syndrome (CRS, dysregulated chemokine and cytokine response) that could be associated with disease severity (29–35). Infected

individuals usually develop first symptoms within five to six days after exposure. Patients with severe illness usually require hospitalization soon after, followed by shortness of breath and acute respiratory distress syndrome (ARDS) (27). ARDS occurs in three percent of the patients infected with the SARS-CoV-2 wild-type variant and is associated with high mortality (28). A subset of these patients shows signs of cytokine release syndrome (CRS) with lymphopenia and excessive release of pro-inflammatory cytokines such as IL-6 and TNF-α (36,37). CRS–induced ARDS was also observed in the previous betacoronavirus outbreaks of SARS-CoV and MERS-CoV.

Infectivity peaks at the time of disease onset or within the first week of illness (27). Viral loads can be quantitatively assessed by real-time reverse transcription-polymerase chain reaction (qPCR) of nasopharyngeal fluids (38).

8.2.2. Immunopathogenesis – relevance of Th1 cells

It has been shown that SARS-CoV-2 infection leads to lymphopenia (31,37,39,40), decreased functional diversity of $CD4^+$ T cells (41) and in severe and critical cases uncontrolled release of pro-inflammatory cytokines – especially a prominent Th1 and Th2 cytokine profile (35,44). Therefore, there is mounting evidence that SARS-CoV-2 acts on lymphocytes, especially T lymphocytes. To understand the pathogenesis of COVID-19, more information on the host immune system is necessary. The role of T cell immunity and the mechanisms that lead to CRS, are currently unknown.

As it is the case with many viral infections, SARS-CoV2 can lead to lymphopenia, especially in severe cases (42,43). More precisely, it has been shown that the relative amount of lymphocytes can be lower than 20% in severe cases (43) and affects B, T, and NK cell numbers (37). Therefore, lymphocyte counts could be used as a marker for disease severity and prognosis in COVID-19. However, lymphocytes are not only reduced but also highly activated and dysfunctional. It has been reported that CD38 (44) but also CD69 and CD44 are highly expressed on both $CD4^+$ and $CD8^+$ T cells, indicating an activated phenotype (44). In HIV infected individuals CD38 expression is associated with decreased viral loads and protects cells from death (45). Furthermore, in a COVID-19 case report activated $CD4^+$ and $CD8^+$ T cells were

increased before symptom relief (46). Therefore, this marker might be used to predict disease outcome.

T cell exhaustion has also been reported in COVID-19. In general T cell exhaustion results in dysfunctional T cells that arise as a consequence of chronic infections or cancer and lead to poor effector functions (47). For example the exhaustion markers programmed death protein-1 (PD1) and T cell immunoglobulin and mucin domain-containing protein3 (Tim3) have been reported to be increased in COVID-19 patients which may be induced by persistent T cell stimulation of the SARS-CoV-2 virus (48). High cytokine levels is another key characteristic of especially severe COVID-19. These patients uncontrollably release a huge amount of different cytokines, also known as CRS. This includes but is not limited to the pro-inflammatory cytokines IL-1β, IL-2 (secreted by Th1 cells), IL-6, IL-7, IL-8, IL-10, granulocyte macrophage colony stimulating factor (GM-CSF), IFN-γ, TNFα, and the chemokines interferon-inducible protein 10 (IP10) and chemotactic protein 1 (MCP1) (49,50). Elevated levels of IL-1β, IFN-γ, IP10, and MCP1 are suggested to activate Th1 responses, whereas IL-4 and IL-10 lead to Th2 responses (31).

Th1 cells represent one of the major subsets in the coordination of an infection and the Th polarization can also determine disease outcome (51). More precisely, a prominent Th1 response is protective and linked to good prognosis and viral clearance (52). Therefore, therapies that promote Th1 responses/polarization could decrease disease severity. Furthermore, SARS-CoV-2 spike protein specific T cells also show a Th1 cytokine profile (53) and COVID-19 mRNA vaccines also elicit a Th1 immune response including robust IFN-γ expression and can therefore protect against severe and criticalCOVID-19 disease. Especially, as IFN-γ has been shown to be one of the key cytokines in antiviral responses. IFN-γ can inhibit the replication of DNA and RNA viruses including SARS-CoV and might therefore reduce viral loads, whereby individuals might have a less severe form of the disease as they could control and eliminate the virus easier (54).

However, at the beginning of the pandemic a comprehensive resource of T cell response to SARS-CoV-2 did not exist, although the information is mandatory for vaccine development (55), identification of therapeutic strategies, and to understand the consequences of SARS-CoV-2 infection. Therefore, we established detailed T

cell profiles, to identify the T cell specific immunopathogenesis of SARS-CoV-2. Different studies reported that COVID-19 patients not only have reduced T cell counts, but also show an altered T cell distribution (42,56).

8.3. Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a systemic chromic autoimmune disease with a prevalence of 0.5-1% (57) in white individuals and 5-6% in native American populations (58). RA patients suffer from painful and destructive inflammation of their joints and the majority of patients develop autoantibodies such as the rheumatoid factor (IgM most common, found in 60-80% of RA patients) or anti-citrullinated protein antibodies (ACPA, detected in 70-90% of RA patients) which defines the seropositive group of patients (59). However, a subgroup of patients (seronegative patients) do not develop these autoantibodies, which highlights the complexity of this disease. Due to intra-, or inter-individual heterogeneity, diagnosing RA is a very individualized process that is based on classification criteria (European League Against rheumatism and American College of Rheumatology classification criteria), which include clinical manifestations and serological assays. The clinical manifestations include, joint swelling, morning stiffness, tenderness, and pain. Disease activity can be measured by different clinical scores. Briefly, these scores are evaluated by distinct formulas that integrate several variables like swollen and tender joint counts, C-reactive protein levels (marker for systemic inflammation) (60).

8.3.1. Pathogenesis – risk factors, development and progression

Risk factors: There are several risk factors for the development of RA, including genetics, female sex (female/male ratio 3:1 (57), this might in part be explained by genetic factors and hormones although the studies are controversial (61)), and environmental factors such as smoking, obesity, and changes in microbiome. The exact pathogenesis of RA is still elusive. Underlying genetic factors and the strong association of RA with certain class II human leucocyte antigen (HLA) molecules, such as HLA-DRB1*004 (HLA-DR4) that contain the share epitope, suggest defects in $CD4^+$ T cell function and their interaction with B cells and antigen-presenting cells (62,63). The shared epitope, encoded by HLA-DRB1*04 and HLA-DRB1*01, is a

specific five amino acid motif that is associated with the risk of developing severe RA (64,65). Furthermore, epigenetics, especially DNA methylation and histone acetylation, may play a role in RA development (66). Understanding how these risk factors contribute to RA development may lead to improvement of understanding this incurable disease.

Development of RA: Development of RA is based on a predisposing genotype and can for example be initiated by other genetic (post-translational modifications) and environmental factors. The disease course usually starts with a preclinical phase, which begins years before clinical manifestation (Figure 2A). Autoantibodies and pro-inflammatory cytokines can be detected up to ten years prior clinical disease onset (60). Examining the synovium of individuals at risk for RA development, more precisely subjects that produce autoantibodies but do not show any clinical sings of RA, revealed that there is no to little infiltration of inflammatory cells or synovitis in these individuals. However, subtle $CD3^+$ T cell infiltrates may be found in the synovium, that tend to correlate with those who subsequently developed arthritis. Therefore, immune cell infiltration of the synovium occur relatively late in the pathogenesis and initial responses might take place in other organs (e.g. lung) (67). Autoantibody production alone is not sufficient to initiate the disease, other factors are needed. RA can for example be initiated by a minor trauma, viral infection, cigarette smoke or microbiota leading to post-translational modification (citrullination) of distinct proteins including for example histones, collagen, fribronectin, fibrinogen, and, vimentin (60,68,69). Citrullination but also other modifications like acetylation or carbamylation alter these peptides resulting in targets for autoantibodies (70,71).

Early RA usually affects the smaller joints and can spread to the larger joints as the disease progresses. Furthermore, immune cells, especially $CD4^+$ T cells and macrophages start to infiltrate the synovium, expression of matrix-degrading enzymes is increased, and plasmablasts expand (60). As the disease progresses T cells become much more polyclonal, consequently it is difficult to detect arthritogenic T cells in the established disease (72). The major characteristics of the established disease include, infiltration of inflammatory cells into the synovium leading to synovial hyperplasia, formation of an invasive pannus destroying adjacent

cartilage and bone, and increased expression of RANKL whereby osteoclastogenesis is increased (73).

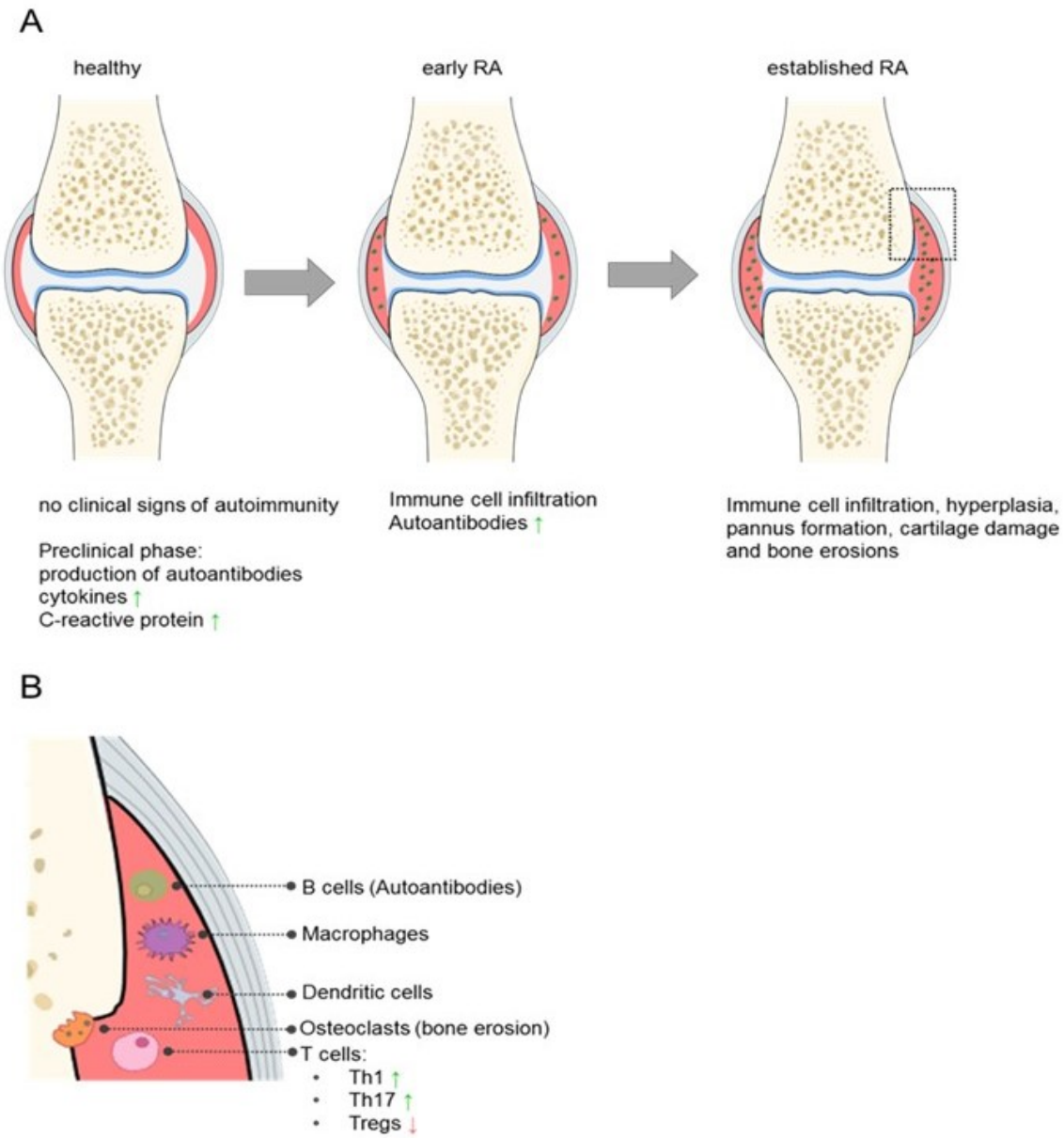

Figure 2: **Pathogenesis of RA** (A) Schematic summary of disease progression from healthy or asymptomatic patients to early and established RA. The dashed rectangle marks the area of figure (B), which illustrates the key immune cells implicated in RA that lead to bone erosions.

Pathogenesis: There are different comorbidities reported in RA, including cardiovascular and lung diseases. Cardiovascular disease is also the most common cause of death in RA patients (74). However, the joint or more precisely the synovium, is the main target tissue of autoreactive immune cells. The main role of the synovium is to protect the cartilage by producing or providing lubricants and nutrients. The healthy synovium consist of a thin layer of fibroblast and macrophage-like synoviocytes, fibroblasts, and adipocytes. The first pathological changes of the synovium include two main changes: I) expansion due to synoviocytes, leading to an increased expression of proteases, and cytokines, including matrix metalloproteinase, IL-1, IL6 and TNF and II) infiltration of adaptive immune cells (60). Lymphocytes and antigen presenting monocytes, and macrophages belong to the key cellular components in RA. Infiltrating lymphocytes dominate the sublining layer of the synovium of recently diagnosed patients (75,76). Furthermore, in patients with an established disease, lymphocytes and plasma cells predominate the areas of dense cell infiltration. Infiltrating B cells play an important role as they produce large amounts of autoantibodies (76) and T cells orchestrate the inflammatory responses and therefore play a major role. Synovial macrophages and monocytes promote inflammation by secreting cytokines, chemokines, and degrading enzymes, but also by activation of other cells. In addition they are also associated with disease severity and therapy response (77,78). As mentioned elsewhere, immune cell infiltration leads to the formation of an invasive pannus that finally damages cartilage and bone. Receptor activator of nuclear factor-κB ligand, produced by infiltrating T cells, activates osteoclasts that leads to bone erosions by degrading the mineralized bone via proteases (60). The key immune cells that infiltrate the erosive joint are depicted in Figure 2B.

8.3.2. Relevance of T cells especially Th1 cells in the pathogenesis of RA

In Rheumatoid arthritis, T cells, especially $CD4^+$ T cells dominate the infiltrated and inflamed synovial membrane, whereas $CD8^+$ T cells occur less frequent and B lymphocyte amounts vary between patients and can be very low or absent in some of them (79). However, it is generally known that B cells support RA through for example autoantibody production and activation of T cells by presenting the relevant antigen (80). Beside B cells, there are other antigen presenting cells, including

macrophages and dendritic cells, but also fibroblast-like synoviocytes that are important partners of the infiltrating T lymphocytes to support the chronic inflammation present in these patients (79).

The exact role of T cells, in the pathogenesis of RA is still not completely understood, but there are numerous studies that support their crucial role in driving the disease. In a humanized mouse model of synovitis inflammation was suppressed after eliminating most of the infiltrating T cells, whereas adoptive transfer of $CD4^+$ T cells resulted in increased synovial inflammation (81). The strong association with certain MHC class II genes (HLA-DR4) and the presence of abundant synovial T, especially $CD4^+$, cells support their key role in RA. Furthermore, in a mouse model of inflammatory arthritis, it has been shown that the initiation of arthritis was dependent on $CD4^+$ T cells but not $CD8^+$ T cells or B cells (82). $CD4^+$ cell depletion reduced diseases severity in the collagen-, and also antigen-induced arthritis model by suppressing the autoantibody production (83). These examples highlight the importance of $CD4^+$ T cells in RA. In patients with recent RA onset, T cell numbers correlated with severity of joint damage (84).

The distinct $CD4^+$ effector subpopulation, differ in distribution and function. However, many studies reported an essential Th1 contribution in the pathogenesis of RA. Although there is mounting evidence that Th17 have a crucial role, it has been shown that Th1 clearly dominate the joints of RA patients (85). Breedveld and co-workers studied the effects of CD4 depleting antibody therapy in patients with early RA (86,87). They did not identify any improvement in clinical parameters and diseases activity but measured a substantial decrease in circulating $CD4^+$ T cell numbers (86) and synovial inflammation (87). Furthermore, unspecific $CD4^+$ T cell depletion may lead to a dysregulation in the immune system as these cells exert both inflammatory but also suppressor functions. Therefore, there are attempts to block distinct $CD4^+$ cell subsets including for example IFN- γ, which is produced by Th1 cells. Sigidin et al. reported improvement following seven days of anti IFN-γ antibody treatment (88), whereas another more recent phase two clinical trial was terminated as it did not meet the endpoint of the first phase (89). Therefore, targeting IFN-γ in RA seems to be unsuccessful.

However the data on the role of IFN-γ is quite controversial as some mouse studies also identified a protective role for IFN-γ. Therefore, researchers evaluated the

efficacy and safety of IFN-γ treatment and identified clinical improvement following IFN-γ treatment. However, the study cohort was very small as only 16 patients were included. In addition, fever was a very common side effect as it occurred in 88% of treated patients, whereas chills and fatigue was less common (90). Veys et al. concluded from their data that IFN-γ was well tolerated in their patient cohort, but they did not identify any clinical benefit compared to the placebo group (91).

Taken together, further studies are needed to fully understand the pathogenesis of RA and to identify the arthritogenic immune cells.

8.3.3. Conventional mouse models to study the pathogenesis of RA and their limitations

Translating basic immunology from animal models to human has been challenging with only little success, especially as the immune system between humans and rodents differ significantly.

To study the pathogenesis of RA there are numerous different mouse models available. By using these models researchers were able to generate very important information on RA pathogenesis including the role of T cell function in this disease (92). However, each addresses only specific aspects of the disease and they do not fully recapitulate the human immune system. For example, it is generally assumed that T cells are important key players in the pathogenesis of RA (93–96), nevertheless in collagen antibody-induced arthritis (CAIA) mice, it is possible to induce arthritis in the absence of T cells (97). Furthermore, using this mouse model, it has been shown that arthritis severity is dependent on T-bet, which is the master regulator of Th1 cells (98). Th1 cells produce, IFN-γ and different studies reported a pathogenic function. The arthritis development in the proteoglycan-induced model is Th1 , IFN-γ (99) and IL-17 independent (100), although synovial T cells represented a minority within the inflamed joint (101). IFN-γ dominated over IL-4 and IL-10 levels and the IFN-γ to IL-4 ratio peaked at disease onset. Arthritis development can be prevented in these mice by treatment with Th2 cytokines resulting in reduced IFN-γ expression (99). However, it has been shown that depletion of T cells in the peripheral blood of these mice did not diminish joint inflammation (101). In CIA mice neutralization of

IFN-γ by antibodies led to acceleration of the disease and increased IL-17 (produced by Th17 cells) levels (102).

Numerous therapies worked well in mice, but failed in human trials or even led to unforeseen harmful side effects. For example interleukin (IL) 1 and 17 inhibitors abrogate arthritis in mouse models of RA but show only limited efficacy in RA patients (103–105). In total more than 80% of effective and safe new therapeutic interventions which were tested in mice, fail in human trials (106,107). This underlines the limitations of conventional rodent models of RA and raises the question if we can transfer the mouse data to the patients. Especially as, human and mouse differ in both adaptive and innate immune system, including neutrophils/lymphocytes balance (108), Fc receptor and Ig isotypes expression (109),regulation and development of B and T cells (110). However, animal model that accurately mirror the human diseases are needed to get a better understanding of the disease and to perform in-depth analysis on for example cell interactions (by e.g. cell depletions), genetic manipulations, cell homing, and testing of novel therapies (92). Consequently, current research would profit from a humanized mouse model that mirrors the patient's specific pathogenic immune processes, which lead to this painful and incurable disease.

8.3.4. Development of humanized mice

Humanized mice belong to the most promising strategies in basic research to analyze the human immune system *in vivo* without putting patients at risk. These mice are highly immunodeficient and therefore powerful pre-clinical tools to study various human diseases. Humanized mice are therefore defined as immunodeficient mouse strains that are humanized by engraftment with cells (PBMCs or hematopoetic stem cells), tissue (synovium, fetal tissue, thymus), or introduction of human transgenes. There are different mouse strains available, but this work will only focus on humanized non-obese diabetic (NOD) *scid* gamma (NSG) mice.

The scid mutation (protein kinas DNA activated catalytic polypeptide $^{\text{severe combined immunodeficiency}}$, $Prkdc^{scid}$) was discovered first but engraftment rates of human cells were low. Crossing the *scid* mutation onto NOD mice resulted in lower mouse natural killer (NK) cell activity and higher engraftment. However, mouse

experiments were limited by the short lifespan on these mice. Engraftment rates of humanized mice were greatly improved, by homozygous mutations at the IL-2 receptor γ-chain locus (*IL2rg*). NOD *scid IL2rg*$^{-/-}$ have impaired B and T cell development and NK development is completely prevented (111). As a consequence no lymph nodes are formed in these mice (112)

Engraftment rates, and human immune cell distribution, vary between the different humanization strategies. Different injection routes have been used for hematopietic stem cells and PBMC transfer, including intravenous (iv), intraperitoneal (ip), subcutaneous (sc), and intrahepatic (ih) injections. Each differ in humanization rate, for example hematopoietic stem cell transfer efficiencies of iv injected mice were increased compared to ip injections (111). Andrade *et al.* developed a mouse model of systemic lupus erythrometrose by injecting PBMCs from patients into immunodeficient BALB-RAG-2$^{-/-}$IL-2Rγ$^{-/-}$ mice. Consequently these mice develop many clinical features of this autoimmune disease (113). Beside cell transfer, it is also possible to implant human tissue. Weyand and co-workers, developed a humanized mouse model of synovitis (81). Briefly, they implant synovial tissue of RA patients subcutaneously into NSG mice. In addition to engraft the human tissue, they also perform PBMC injections seven days after tissue transplantation. Moreover, there are different humanized mouse models to study the pathogenesis of infectious disease or cancer available (111,112). However, so far a humanized and therefore patient-specific mouse model of RA does not exist but would be crucial to get new insights into the pathogenesis and to test new therapeutic interventions.

The currently available mouse models also have distinct limitations and some of them do not fully recapitulate a functional human immune system. Transgenic mice for example, help to understand the importance of genetic risk factors that are associated with the specific disease. However the disease is still mediated by cells of murine origin (114). Stem cell engraftment of NSG mice will lead to good engraftment rates and differentiation into many hematopoietic lineages (111), but the T cell responses will still be restricted to murine major histocompatibility complex (MHC) (114).

9. Aims of the study

The purpose of this study was to improve the understanding of Th1 cells in different human diseases. We therefore, focus on Th1 cell responses in an infectious disease (COVID-19) and autoimmune disease (RA). Therefore, the approach of this study was to: I) identify T cell response in patients with acute COVID-19 and II) improve our understanding of how arthritis is initiated and to identify the arthritogenic immune cells. Therefore, we aim to comprehensively profile the T cell composition of hospitalized COVID-19 patients generate new insights into the pathogenesis of RA by developing a novel humanized mouse model. This model describes how to transfer immune cells, predominantly Th1, of the peripheral blood of RA patients, to mice to initiate a patient specific inflammatory joint disease and therefore study the pathogenesis of RA *in vivo* on a human immune system. We believe that NOD, SCID, interleukin- 2 receptor γ knockout (NSG) mice, which express the human HLA-DR4, instead of mouse-MHC class II (NSG-AB^0 DR4) represent the ideal platform for this approach.

To assess these aims we developed following research questions:

1. How do T cell responses of hospitalized COVID-19 patients deviate from healthy controls? – focusing on the antiviral Th1 cells.
2. Are immune cells of the peripheral blood of RA patients capable of initiating inflammatory joint disease in immunodeficient mice?
3. Do Th1 cells dominate the human immune cell composition in this mouse model and do they regulate disease severity?
4. Does prophylactic abatacept treatment prevent the inflammatory joint disease in these mice

<u>Ad 1: Th1 response in severe to critical COVD-19 patients</u>

Here we suggest that we will identify significant differences between the healthy and COVID-19 donors. Especially, as Th1 belong together with CD8 T cells to the antiviral subsets. Therefore, we aim to profile the T cell content of peripheral blood in both cohorts using flow cytometry.

Ad 2: Identification of arthritogenic immune cells within the peripheral blood of RA patients

We believe that arthritogenic T cells, which can be found in the peripheral blood of RA patients, can initiate arthritis in immunodeficient NSG mice. Therefore, we decided to combine two humanization strategies. Thereby we could overcome some of to the mentioned limitations and subsequently generate a mouse model that mimics the patients specific pathological mechanisms. NOD, SCID, interleukin- 2 receptor γ knockout (NSG) mice represent the ideal platform for this approach, due to their lack in functional T, B and natural killer cells, resulting in a highly immunodeficient model organism (111,115). Furthermore, mice with additional transgenic expression of human HLA-DR4, instead of mouse-MHC class II (NSG-AB^0 DR4) have become available (115). This genetic modification reduces the occurrence of xenogeneic graft-versus-host disease (GvHD) and improves the function of HLA-DR4 positive human CD4$^+$ T cells (116,117). Given that 45% of RA patients are positive for HLA-DR4 (118) and RA is considered to be a disease initiated and driven by CD4$^+$ T cells, these mice represent an ideal platform for the establishment of a patient-specific, humanized mouse model of RA.

Ad 3: Regulation of disease activity by altering dominate immune cell frequency

Th1 belong together with Th17 and Tregs to the key subsets in RA. Therefore, we aimed to identify which of these cells dominate the human T cells in the engrafted mice using multicolor flow cytometry. Conventional mouse models demonstrated, that Th1 cells have a role in arthritis, especially in the initiation.

Ad 4: Rescuing the arthrtitogenic effect by prophylactic abatacept treatment

We suggest that T cells play a major role in our mouse model. Therefore, abatacept might prevent arthritis development by blocking optimal T cell activation.

10. Material and Methods

10.1. Study approvals

All experiments involving human subjects were carried out according to Austrian laws, the declaration of Helsinki and the principles of good scientific practice. Approval of the ethics committee of the Medical University of Graz (32-434 ex 19/20, 26-599 ex 13/14, and EK-28-016 ex 15/16,) has been obtained. Mouse experiments were approved by the Austrian Ministry of Science and Economy (BMWF-66010/0053-WF/II/3b/14). Furthermore, early endpoints have been chosen to avoid unnecessary animal distress in the *in vivo* experiments.

10.2. COVID-19 study

10.2.1. Study design

Retrospective analysis was conducted of twenty COVID-19 hospitalized patients with severe to critical illness. Additionally, two age-, and sex matched healthy controls (HC) were matched to each COVID-19 patient. We obtained medical history, demographical data, and COVID-19-specific medication through electronic medical records. Exclusion criteria for the HC were pregnancy, infectious and autoimmune diseases, acute or chronic diseases associated with organ damage, increased C-reactive protein levels, neoplasia (present or past), and severe anemia (Hb <9 mg/dl). Detailed T cell phenotyping was performed using multicolor flow cytometry.

10.2.2. Flow cytometry and T cell phenotyping

Staining was performed immediately after blood draw and measured at BD Canto II cytometer (Becton Dickinson). Compensations was performed using single stains and data was analyzed using FloJo software (Treestar, San Diego USA).

Blood was collected in EDTA coated tubes. Cells were stained for 15min using fluorochrome-conjugated anti-human Abs against CD3, CD4, CD8, CD197/CCR7, CD127, CD28, CD25, and CD45RA or CD3, CD4, CD8, CD189/CXCR3, CD196/CCR6, CD194/CCR4, and CD38. Followed by red blood cell lysis using BD Lysing solution (Becton Dickinson, Heidelberg, Germany), fixation/ permeabilization, and 15min intracellular staining using ant-Ki67.

10.2.3. Clinical laboratory measurements

RT-PCR was used to assess viral loads of SARS-CoV-2 using the cobas SARS-CoV-2 test (Roche Molecular Systems, Branchburg, NJ) for use on the cobas 6800/8800 system. Cycle threshold (Ct) values targeting a unique SARS-CoV-2 region (ORF1a/b) were used as a proxy for viral loads. Furthermore, ferritin, CRP, plasma IL-6, and lymphocyte and T cell counts were performed at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz.

10.2.4. Statistics

Distribution of data was tested using Kolmogrov-sminrnov normality test. Data was corrected for outlier using the robust regression and outlier removal method (Q=1). Two independent groups were tested by Mann-Whitney U tests and multiple groups were compared by Kruskal-Wallis and one-way ANOVA with Dunn posttests. Wilcoxon signed-rank tests were performed to study the effects of treatment (paired samples, prior/posttherapy). Correlation analysis is based on Spearman to determine the relationship between IL-6 and antiviral subsets. Cluster analysis was performed using R (https://www.R-project.org/). Briefly, frequencies of all cases (40 HC and 20 COVID-19) were transformed to decrease the variance between relative cell counts of different T cell populations (function “scale” in R). Subsequently, dimensional reduction analysis via Principal Component Analysis (PCA) was performed (119). Hence, the most relevant T cell populations were identified. Transformed frequencies of 18 T cell subsets were found to have the most influence on PC1 and PC2 and were used to calculate a dissimilarity matrix between individuals (Euclidian distance matrix). Unsupervised hierarchical clustering was calculated based on Ward’s minimum variance method (120). Transformed frequencies of T cell subsets and clustering results were summarized in a heatmap (121). Statistical analysis including scatter blots, and correlation analysis were performed using SPSS software version 26 (IBM, New York, NY) and GraphPad Prism software version 8.

10.3. Mouse model of Rheumatoid Arthritis

10.3.1. Study design

The humanization experiments of the NSG-DR4 mice were done at the Medical University Graz. Therefore, we injected naïve T cell depleted PBMC by iv and ip injections into twelve- to eighteen-week-old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} *H2-Ab1*^{tm1Doi} Tg(HLA-DRB1)31Dmz/SzJ (NSG-AB^{0} DR4) mice, obtained from Jackson Laboratory.

All Rheumatoid arthritis patients fulfilled the Rheumatoid Arthritis classification criteria according to the European League Against rheumatism (EULAR) and American College of Rheumatology (ACR) criteria from 2010 (122). Neoplastic disease, acute or chronic infectious disease, pregnancy, significant anemia (Hb <9mg/100mL), and B cell depletion therapy lead to exclusion. Clinical information including gender, age, seropositivity, positive HLA-DR4 status, and medication usage were assessed using medical records. Healthy, HLA-DR4 positive individuals were used as controls. All individuals of the healthy control group were recruited via the Department of Blood Group Serology and Transfusion Medicine, Medical University Graz Austria.

10.3.2. Preparation of human PBMC

Blood samples of patients were obtained and subjected to standard Ficoll density centrifugation (histopaque 1077, sigma). Therefore, blood was diluted with PBS, gently layered over the density gradient medium and centrifuged for 30-40min at room temperature without using the beak. After centrifugation the PBMCs (second layer) were removed using a Pasteur pipette and added to PBS. To reduce the occurrence of GvHD, PBMCs were depleted for naive T cells using magnetically labeled beads. Briefly, this was done via a two-step procedure – I) separation of CD3 positive/negative cells using the REAlease CD3 MicroBead Kit (Miltenyi, Bergisch Gladbach, Germany), II) labeling of CD3 positive fraction using CD45RA MicroBeads (Miltenyi, Bergisch Gladbach, Germany) to deplete the naive T cell fraction ($CD3^{+}CD45RA^{+}$). Both protocols were conducted based on manufacturer's protocols, only the amount of reagents were scaled up by 50%.

1.1.1. Humanization of NSG-DR4 mice

Only $CD3^-$ and $CD3^+CD45RA^-$ cells were injected into the NSG-DR4 mice. Due to the long-term engraftments of 10 weeks, depletion of naïve T cells prior injection is a crucial step, as they would lead to the development of GvHD in this model. After depletion, cells were washed in 2% BSA-PBS and directly injected. Sequential transfer was performed to induce engraftment. Therefore, cell dose was split in two injections. First injection 10 Mio cells, second (three weeks postinjection) 5Mio cells. Both were administered intravenous (tail vein) and intraperitoneal. Arthritis development, weight, and general health state was monitored during the whole experiment. Clinical assessment of arthritis development was evaluated by checking joint swelling, redness of joints, and grip strength. Furthermore, efficiency of engraftment was measured *post-mortem* and during the experiment by flow cytometry analysis.

10.3.3. Th1 polarization

In addition to the standard humanization protocol as described above, $CD3^+CD45RA^-$ cells were also cultured Th1 polarization media. Therefore PBMC were isolated, depleted for naïve T cells, and before first injection, 2Mio $CD3^+CD45RA^-$ cells were cultured in AimV media containing IL-12 and T cell transact. Same relative amount of $CD3^-$ fraction was discarded. Remaining cells were mixed together for cell injection (10Mio per mouse as described above). Seven to days post-injection, 2.5Mio of Th1 polarized $CD3^+CD45RA^-$ cells were injected Iv/ip (50:50) into the same mouse to boost the T cell engraftment.

10.3.4. Flow cytometry

Staining was performed immediately after blood draw or mouse dissection and measured at BD Canto II cytometer (Becton Dickinson). Compensations was performed using single stains and data was analyzed using FloJo software (Treestar, San Diego USA).

Blood/ tissue preparation: Whole blood was drawn (in mice via vena facialis), washed in PBS, lysed (Becton Dickinson, Heidelberg, Germany), washed in PBS containing 2% FCS, and stained. After dissection, spleens were transferred immediately into PBS (4°C). For FACS analysis spleens were minced, filtered (100µm and 40µm cell strainers), lysed (7min, 4°C), washed, and stained.

Humanization efficiency staining: Both blood and splenocytes were stained (30min 4°C) using fluorochrome conjugated antibodies against human CD3, CD4, CD8, CD19, and mouse CD45.

Surface and intracellular T cell panel: Cells were stained (15min 4°C) using fluorochrome-conjugated anti-human antibodies against CD3, CD4, CD8, CD189/CXCR3, CD196/CCR6, CD194/CCR4, and CD38, washed, and incubated for 30 min on ice with fixation/ permeabilization solution (Thermo Fisher, Massachusetts, USA), washed and incubated with anti-Ki67 for 15 min at 4°C.

Surface T and B cell panel: Staining was carried out as described above, without permeabilization. Again, cells were stained (15min 4°C) using fluorochrome-conjugated anti-human antibodies against CD3, CD4, CD8, CD197/CCR7, CD127, CD28, CD25, and CD45RA. For B cell assessment antibodies against CD19, IgD, CD24, CD27, CD38, CD86, CD25, and IgM were used.

10.3.5. *In vivo* CTLA-4 treatment

Mice were humanized as described above. Two NSG-DR4 mice were humanized with each RA donor to compare treated with untreated mice. Prophylactic CTLA4-IgG (Abatacept) was performed. Therefore, per mouse 0.25mg Abatacept was injected subucanteously once a week, starting two weeks after engraftment. Control mice were injected with PBS buffer.

10.3.6. Histologic assessments

Organs including, heart, lung, salivary glands, pancreas, skin, as well as joints of the humanized mice were isolated, fixed (24h 4% PBS bufferd formalin), and paraffin imbedded. Prior paraffin embedding, joints were transferred into a decalcification solution until pliable (12,5% EDTA, pH 7-7.4, about one week at 50°C). In addition to routine HE staining, performed after standard protocols, immunohistochemimstry staining was done to determine the infiltrating human immune cells. Therefore, sections were stained using antibodies against human CD45, CD4, CD8 and CD19 and counterstained using Mayer's hemalum solution. Safranin O and TRAP staining was performed to detect cartilage damage and osteoclasts.

10.3.7. Statistics

Arthritis incidences over time were presented in a Caplan-Meier graph. Arthritis curves were compared using log-rank (Mantel-Cox) test. Flow cytometry data of engrafted human cells are depicted as % of human $CD45^+$ cells, % of human $CD3^+$ cells, absolute cells per spleen, or fold change (normalized on corresponding patient sample). Two unpaired groups were compared using t-test for parametric and Mann-Whitney for nonparametric distributions. Normality was tested using Shapiro-Wilk and Kolmogorov-Smirnov test. 2-way ANOVA was used to assess differences between more than two groups. Sidak and Bonferroni were used as posttest to correct for multiple comparisons. Statistical analysis including the corresponding graphs were performed using Graphpad Prism software version 8. P-values above 0.05 were considered as statistically significant ($*p \leq 0.05$ $**p \leq 0.01$ $***p \leq 0.001$).

11.Results

11.1. Dysfunctional antiviral T cell responses in COVID-19

11.1.1. Overview of the enrollment

We included 20 severe to critical COVID-19 patients and 40 age-, and sex-matched healthy controls in this study. Demographic data including age and sex was gathered from all subjects. The patient cohort contained 25% female and 75% men. 45% had follow up measurements (no significant changes in cell composition between measurements; data not shown), 45% needed ventilation, and 30% succumbed to COVID-19. Table 1 summarizes age, gender, disease severity, COVID19 specific medication, and outcome of each patient.

Table 1 COVID-19 patient cohort characteristics

patient	age	gender	AHT	DM	severity	ICU	Intubation	HCQ	lopinavir/ ritonavir	Tocilizumab	CPT	follow-up measurements	outcome
1	53	male	no	no	critical	yes	yes	yes	no	yes	yes	yes (3)	discharged
2	80	male	yes	no	critical	yes	yes	yes	no	no	no	no	discharged
3	66	male	yes	no	critical	yes	yes	no	yes	no	no	yes (1)	died
4	68	male	no	no	critical	yes	yes	yes	yes	no	no	yes (1)	died
5	78	female	no	no	severe	no	no	no	no	no	no	no	discharged
6	58	female	no	no	severe	no	no	yes	no	no	no	yes (1)	discharged
7	72	male	yes	yes	critical	yes	yes	yes	no	no	no	no	died
8	56	female	no	no	severe	no	no	yes	no	no	no	no	discharged
9	47	female	no	no	severe	no	no	yes	no	no	no	no	discharged
10	80	male	no	no	critical	yes	yes	yes	no	no	no	no	died
11	63	male	no	no	critical	yes	yes	yes	no	yes	no	yes (1)	discharged
12	56	male	yes	no	critical	yes	yes	yes	no	yes	yes	no	discharged
13	67	female	no	no	critical	yes	yes	yes	yes	no	no	yes (1)	discharged
14	74	male	yes	yes	severe	no	no	yes	no	no	no	no	discharged
15	37	male	no	no	severe	no	no	yes	yes	yes	yes	yes (2)	discharged
16	78	male	no	no	severe	no	no	yes	no	no	no	no	discharged
17	80	male	no	no	severe	no	no	no	no	no	no	no	discharged
18	69	male	no	yes	critical	yes	yes	yes	no	yes	no	yes (2)	died
19	69	male	no	yes	critical	yes	yes	yes	yes	yes	no	yes (2)	discharged
20	78	male	no	yes	critical	yes	no	no	no	no	no	no	died

AHT, arterial hypertension; DM, diabetes mellitus; ICU, intensive care unit; HCQ, hydroxychloroquine; CPT, convalescent plasma transfusion.

11.1.2. T cell composition segregate between COVID-19 and healthy individuals

The used gating strategy to phenotype the T cell content is depicted in Figure 3. T cell subsets were identified using different surface markers.

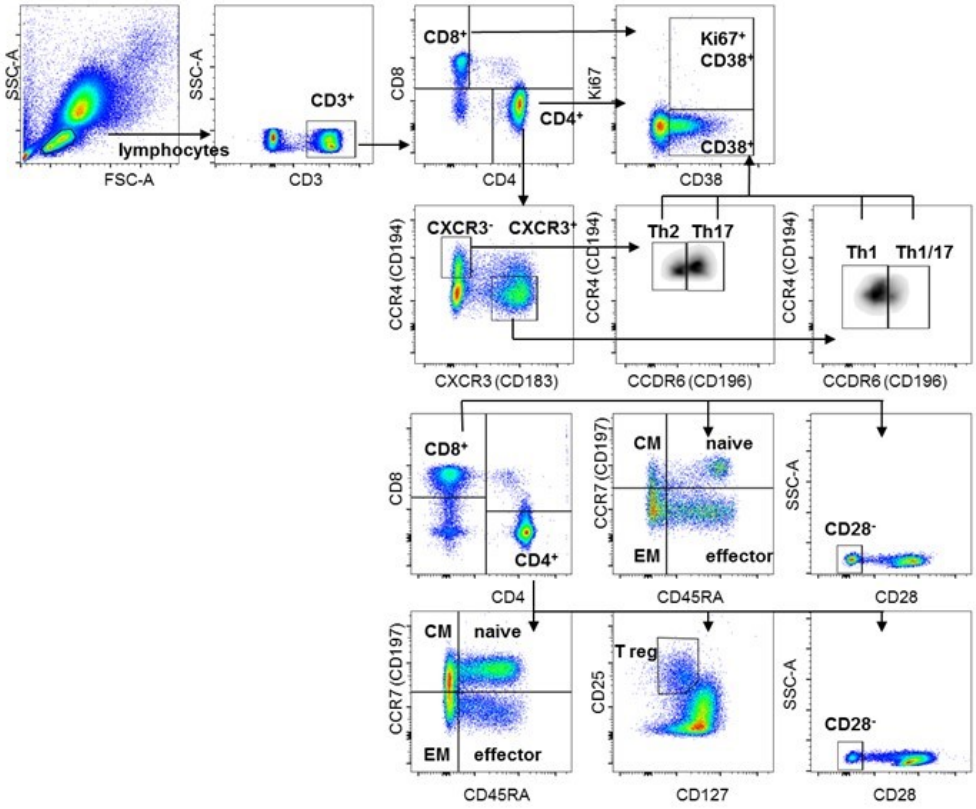

Figure 3 T cell gating strategy. Data was originally published in The Journal of Immunology Rupp J, Dreo B, Gütl K, Fessler J, Moser A, Haditsch B, Schilcher G, Matzkies LM, Steinmetz I, Greinix H, Stradner MH. 2021. T Cell Phenotyping in Individuals Hospitalized with COVID-19. J Immunol. Vol:206. Issue7. Copyright © 2021 by The American Association of Immunologists, Inc. Permission to include the data has been obtained. Representative polychromatic dot blots demonstrating the gating strategy to identify the different T cell subsets.

As reported (37,123,124), COVID-19 patients show decreased frequencies in lymphocytes including decreased B- and T cell counts (Figure 4A-C) . Notably, the percentages of $CD8^+$ T cells was quite variable within the severe group (Figure 4D). Based on the decrease in absolute lymphocyte numbers, we performed in depth analysis only on the frequencies of $CD4^+$ and $CD8^+$ subpopulations. Unsupervised hierarchical clustering revealed a stratification of HC and COVID-19 patients (Figure 4E). We assed 18 T cell subsets that lead to the differentiation of healthy and COVID-19 (**Figure** 5).

Relative numbers of $CD45RA^{+}CCR7^{+}$ naïve, $CD45RA^{+}CCR7^{-}$ effector and memory ($CD45RA^{-}CCR7^{+}$ central memory and $CD45RA^{-}CCR7^{-}$ effector memory) T cell subsets were elevated in HC compare to COVID-19 and $CD8^{+}CD28^{-}$ cells were expanded. Intriguingly, we identified a small subclaster, consisting of patients that succumbed to COVID-19 (four of six) which was characterized by lower naïve $CD4^{+}$ T cell frequencies. Based on these results we further examined the $CD4^{+}$ subsets between the healthy controls, severe, and critical COVID-19 cases Compared to HC, percentages of $CD3^{+}CD4^{+}CXCR3^{+}CCR4^{-}CCR6^{-}$ Th1 were significantly decreased in critical cases, Th17 increased in both severe and critical, and we did not identify any changes in the Treg subset (Figure 4F-I).

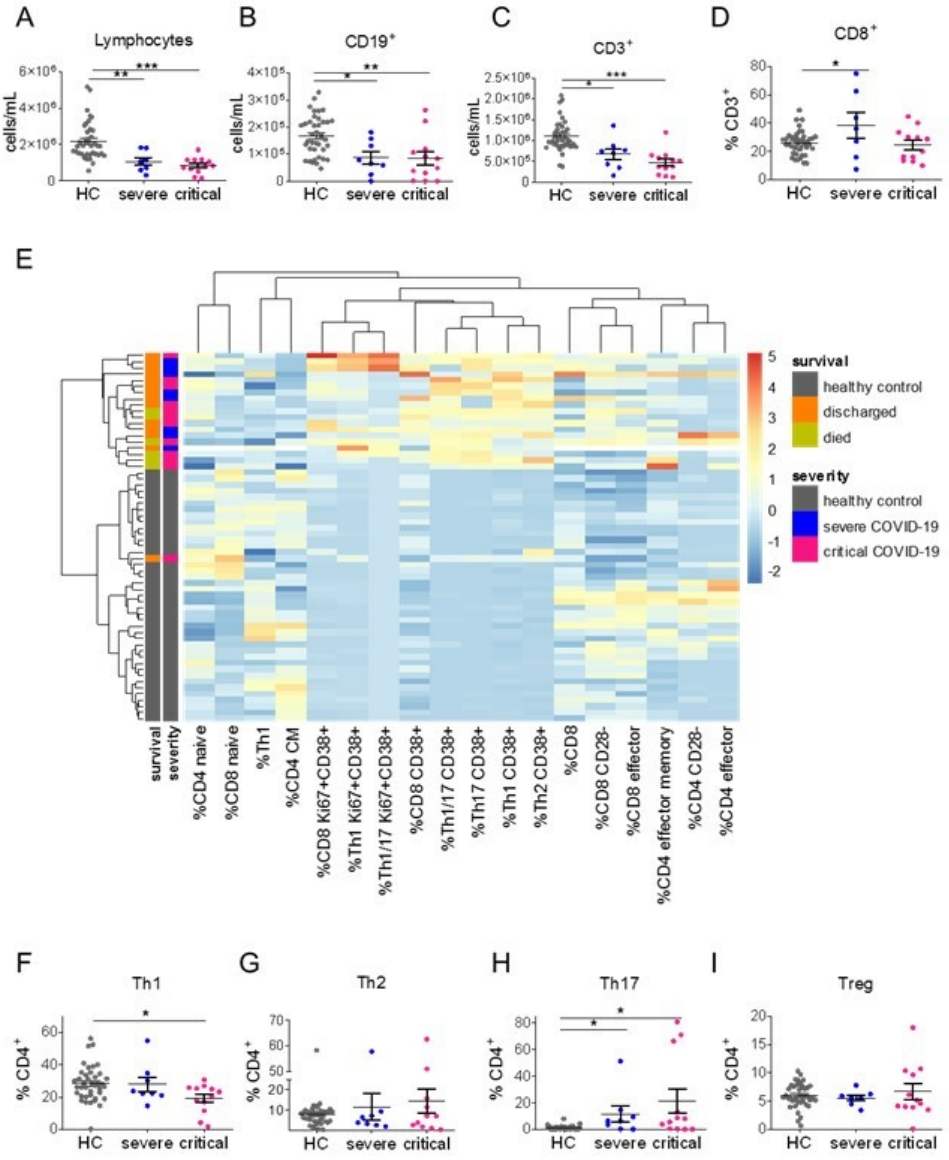

Figure 4 T cell composition differ significantly between COVID-19 and healthy subjects. Data was originally published in The Journal of Immunology Rupp J, Dreo B, Gütl K, Fessler J, Moser A, Haditsch B, Schilcher G, Matzkies LM, Steinmetz I, Greinix H, Stradner MH. 2021. T Cell Phenotyping in Individuals Hospitalized with COVID-19. J Immunol. Vol:206. Issue7. Copyright © 2021 by The American Association of Immunologists, Inc. Permission to include the data has been obtained. (A-C) Absolute cell counts of total lymphocytes, $CD19^+$, and $CD3^+$ cells (D) Frequencies of $CD8^+$ T cells displayed as percentage of $CD3^+$ cells. (E) Unsupervised hierarchical clustering based on the frequencies of 18 cell subsets identified by principal component analysis. Cell frequencies are visualized in a heat map and cohorts were color coded based on survival and health state. (F-I) Th1, Th2, Th17, and Tregs shown as frequencies of $CD4^+$ cells. 20 COVID-19 (8 severe and 12 critical cases) and 40 healthy subjects were included. ANOVA was preformed to compare three groups (Dunns posttest). Each data point represents one individual and data are presented as mean±SEM *P≤0.05, **P≤0.001, ***P≤0.001

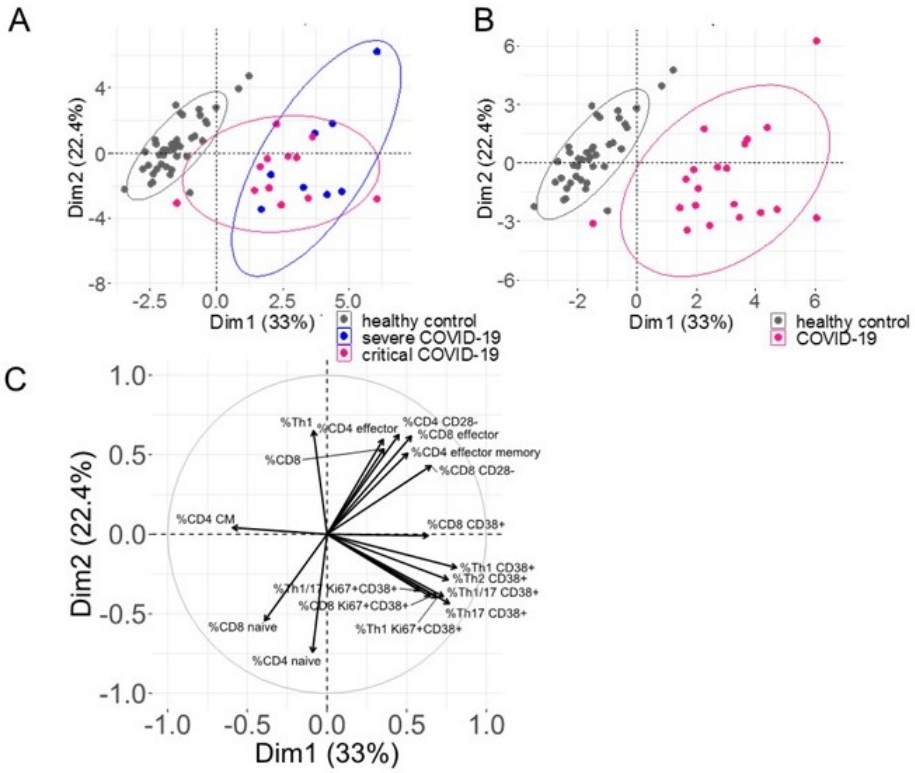

Figure 5 Principal component analysis. Data was originally published in The Journal of Immunology Rupp J, Dreo B, Gütl K, Fessler J, Moser A, Haditsch B, Schilcher G, Matzkies LM, Steinmetz I, Greinix H, Stradner MH. 2021. T Cell Phenotyping in Individuals Hospitalized with COVID-19. J Immunol. Vol:206. Issue7. Copyright © 2021 by The American Association of Immunologists, Inc. Permission to include the data has been obtained. A,B) Scatter plot indicating individual in the first two principal components following variable reduction. Ellipses indicate the 85% confidence interval based on disease severity. C) Represents the variable correlation plot in the first two components, showing the 18 T cell subsets that were used for clustering.

11.1.3. T cell function is altered in SARS-CoV-2 infected patients

To identify differences in T cell proliferation and activation present in COVID-19, we profiled the T cell content using Ki67 and CD38. Increased CD38 expression is associated with immune cell activation and predicts, in association with $CD8^+$ T cells, HIV progression (125). Ki67 is a proliferation marker and measures antigen specific T cell proliferation (126). Compared with matched HC, both markers were significantly elevated in $CD4^+$, $CD8^+$, Th1, and Th2 cells of SARS-CoV-2 infected patients. Pointing out that the largest fold-change was found in the antiviral subsets $CD8^+$ and Th1 (Figure 6).

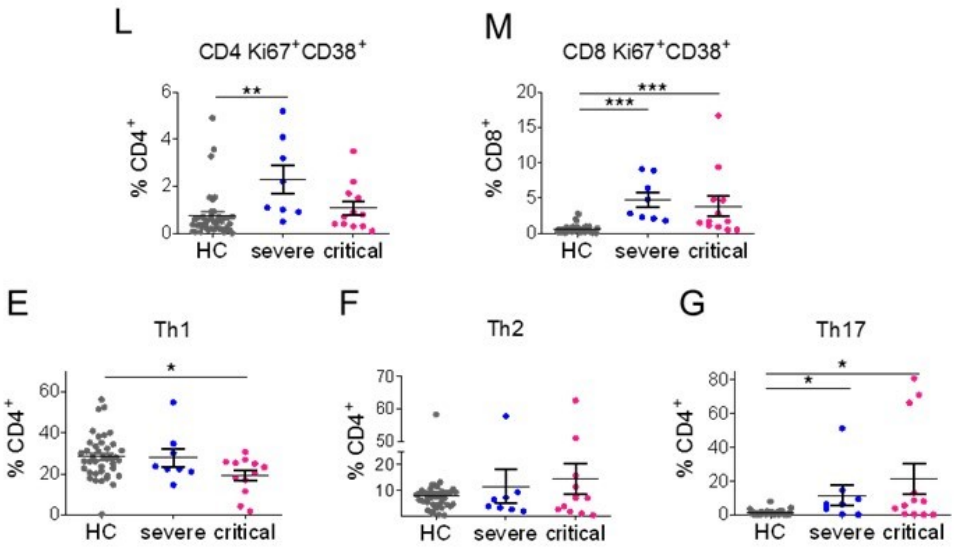

Figure 6 T cell function is altered within SARS-CoV-2 infected patients. Data was originally published in The Journal of Immunology Rupp J, Dreo B, Gütl K, Fessler J, Moser A, Haditsch B, Schilcher G, Matzkies LM, Steinmetz I, Greinix H, Stradner MH. 2021. T Cell Phenotyping in Individuals Hospitalized with COVID-19. J Immunol. Vol:206. Issue7. Copyright © 2021 by The American Association of Immunologists, Inc. Permission to include the data has been obtained. (A-B) Activated and proliferative $CD4^+$ and $CD8^+$ T cells ($CD4^+Ki67^+CD38^+$, $CD8^+Ki67^+CD38^+$) displayed as % of $CD4^+$ and $CD8^+$, respectively. (C-E) $Ki67^+CD38^+$ double positive T helper subsets shown as % of Th1, Th2, and Th17. Forty healthy controls, eight severe and twelve critical cases were included. Data are presented as mean±SEM and each dot represents an independent data point. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

11.1.4. CRS is associated with impaired antiviral defense

CRS is characterized by excessive release of pro-inflammatory cytokines such as IL-6 and TNF-α. Particularly IL-6 is supposed to play a key role in the pathogenesis of this life-threatening syndrome (36,37,127). IL-6 is a pro-inflammatory regulator of T cells and is mainly produced by macrophages. In line with previous reports, we found that disease severity of COVID-19 infections show association with increased plasma IL-6 concentrations (Figure 7A) (37). Therefore, we hypothesized that IL-6 has the potential to regulate anti-viral immune cell function.

It has been shown that IL-6 levels above 80pg/mL predict respiratory failure in COVID-19 patients (128). Analyzing this subgroup, we observed that IL-6 positively correlates with Th1 cell abundance, whereas Th2, Th17, and regulatory T cells showed no correlation. In contrast, frequencies of activated $Ki67^{+}CD38^{+}$ Th1 and $CD8^{+}$ $Ki67^{+}CD38^{+}$ cells as well as total $CD8^{+}$ T cells were diminished with increasing IL-6 concentrations. (Figure 7B-E) suggesting that IL-6 levels regulate activity and proliferation of anti-viral immune cells. Furthermore, we performed *in vitro* experiments to test whether increasing IL-6 concentrations could inhibit anti-CD3/CD28 induced T cell proliferation. However, IL6 treatment did not alter apoptosis, activation, proliferation, or expression of exhaustion markers (PD-1 and CTLA-4) in Th1 or $CD8^{+}$ cells *in vitro* (data not shown). Additionally, we correlated clinical parameters, including duration of symptoms, viral load (Ct of SARS-CoV-2 PCR), oxygen supplementation, CRP, ferritin, and IL-6 with relative abundance of T cell subpopulations. There was no significant correlation with T cell populations and oxygen consumption, ferritin or CRP levels. However, we identified a negative correlation between the Ct-value and central memory $CD8^{+}$ T cells (Figure 7F). Furthermore, plasma IL-6 levels correlated inversely with absolute CD3 cell counts, $CD4^{+}Ki67^{+}CD38^{+}$ and $Th1^{+}$ $Ki67^{+}CD38^{+}$ cells (Figure 7G-I).

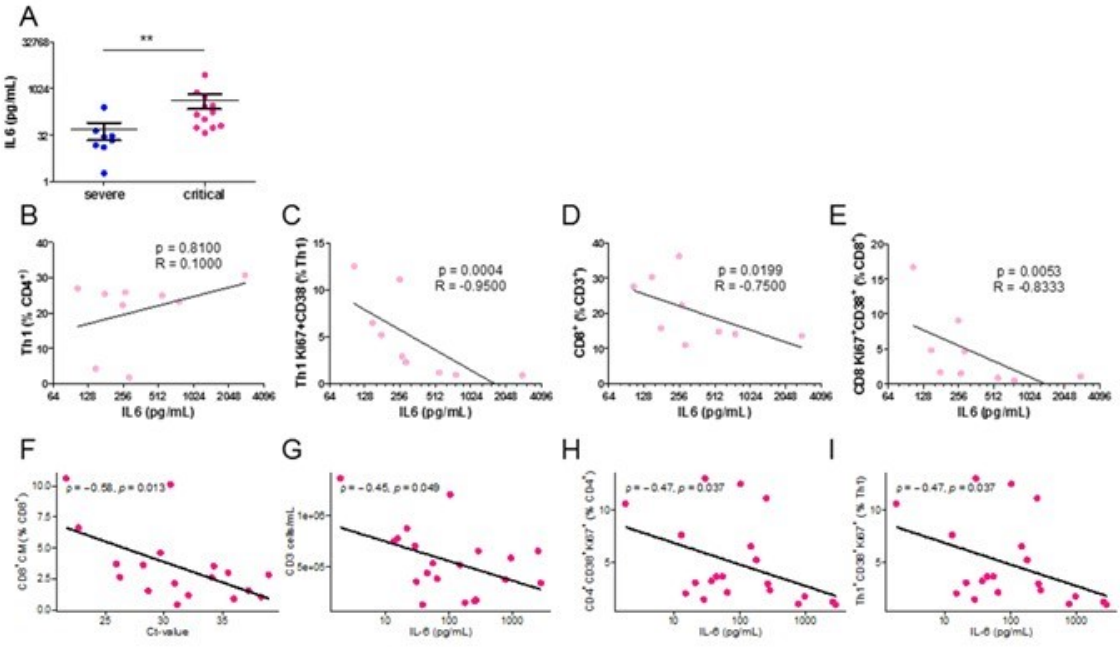

Figure 7 **Increased plasma IL-6 levels negatively predict antiviral defense.** F-I Data was originally published in The Journal of Immunology Rupp J, Dreo B, Gütl K, Fessler J, Moser A, Haditsch B, Schilcher G, Matzkies LM, Steinmetz I, Greinix H, Stradner MH. 2021. T Cell Phenotyping in Individuals Hospitalized with COVID-19. J Immunol. Vol:206. Issue7. Copyright © 2021 by The American Association of Immunologists, Inc. Permission to include the data has been obtained. A) IL-6 levels of severe (n=8) and critical (n=12) SARS-CoV-2 infected patient. Correlations of IL-6 levels above 80pg/mL and (B) Th1, (C) Th1 Ki67+CD38+, (D) CD8+, and (E) CD8+Ki67+CD38+ in COVID-19 (n=9). (F) Ct-values of SARS-CoV-2 and centram memory CD8+ correlate inversely. (G-I) Correlations of plasma IL-6 and (G) T cell counts, relative (H) CD4+Ki67+CD38+, and (I) Th1Ki67+CD38+.Mann-Whitney U was performed to compare IL-6 of critical and severe patients (A) and Spearman analysis for linear correlations (B-I). Data are presented as mean±SEM. **P ≤ 0.01

11.1.5. CRS is associated with impaired antiviral defense

Since it has been shown that *in vitro* blockade of IL-6 during a chronic virus infection resulted in better virus-specific $CD8^+$ T cell response, increased IFN-γ production, and decreased virus loads, we assessed the clinical and cellular effects of IL-6 blockade (Tocilizumab) in patients with COVID-19. The follow-up measurements of our study demonstrate the immunologic trend following IL-6 blockade. Not surprisingly, CRP levels were strongly reduced (Figure 8A). Interestingly, lymphocyte counts already increased five days post therapy start, except the patient who succumbed to COVID-19 (Figure 8B). The antiviral subsets $CD8^+$ and Th1 as well as their activated and proliferative forms, increased in three of four patients

within this time period (Figure 8 C-F). However, a larger sample size and longer time courses are necessary to evaluate significant changes within these cell subsets.

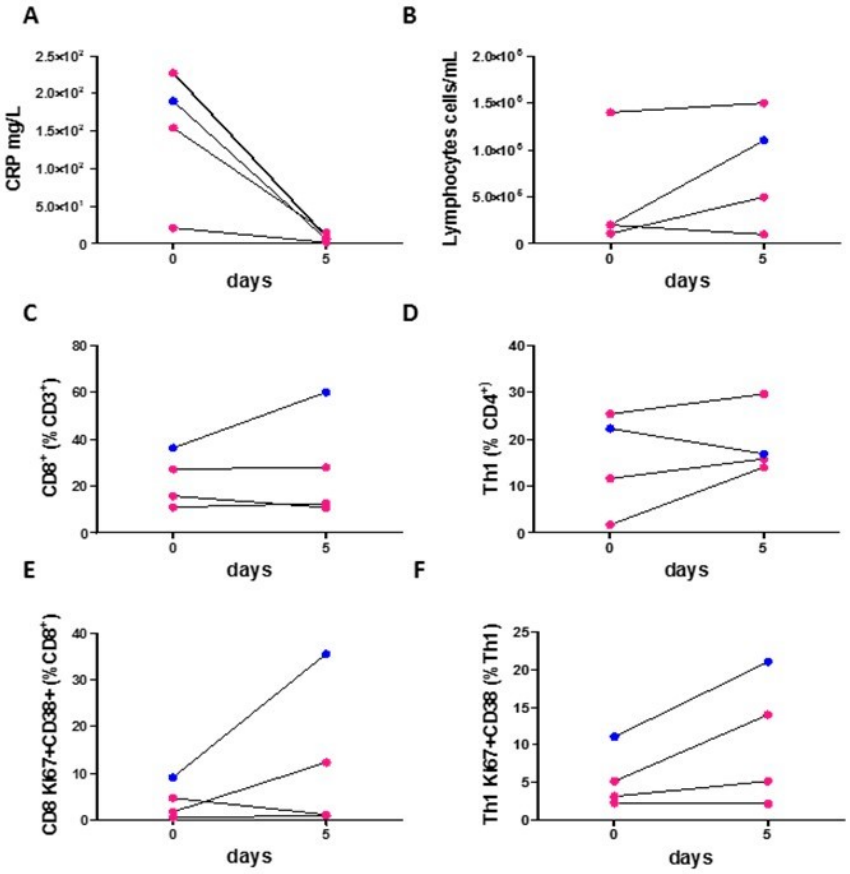

Figure 8 Effect of IL-6 blockade. Data was originally published in The Journal of Immunology Rupp J, Dreo B, Gütl K, Fessler J, Moser A, Haditsch B, Schilcher G, Matzkies LM, Steinmetz I, Greinix H, Stradner MH. 2021. T Cell Phenotyping in Individuals Hospitalized with COVID-19. J Immunol. Vol:206. Issue7. Copyright © 2021 by The American Association of Immunologists, Inc. Permission to include the data has been obtained. Prior and post (5 days) anti IL-6 therapy measurements of (A) CRP levels in mg/L, (B) Lymphocytes counts, and frequencies of antiviral T cell subsets (C) $CD8^+$, (D) Th1, (E) $CD8^+Ki67^+CD38^+$, and (F) $Th1Ki67^+CD38^+$. n=4. Differences between the two time points were evaluated using Wilcoxon signed-rank test. Blue represents severe and pink critical COVID-19 cases.

11.2. Development of a Th1 driven humanized mouse model of RA

Research has been hampered by the lack of appropriate models to study the human immune system. We successfully established a protocol to generate humanized mice to study the pathogenesis of rheumatoid arthritis in vivo (Figure 9A). These mice harbor, without any host preconditioning, a patient-specific human immune system that cause inflammatory joint disease. Of note, arthritis development is significantly increased in mice engrafted with cells from RA patients ($p=0.0068$, Figure 9B), although mice injected with cells from healthy donors show similar humanization rates (Figure 9C). Arthritis development was characterized based on clinical joint swelling and histological hallmarks of the disease including infiltration of inflammatory cells, pannus formation, increased osteoclastogenesis, bone erosions and cartilage damage (Figure 9 D-G). In addition, immunohistochemistry staining revealed infiltration of human CD45+ cells in the area of bone erosions (Figure 9H). Furthermore, we identified bone remodeling, more precisely osteophyte formation, using micro computed tomography analysis of the joints (Figure 9 I-L). We suggest that osteophytes formed due to discontinued inflammation and repair mechanisms, as we did not sacrifice the mice at peak of disease (joints were collected six weeks after first clinical signs of arthritis).

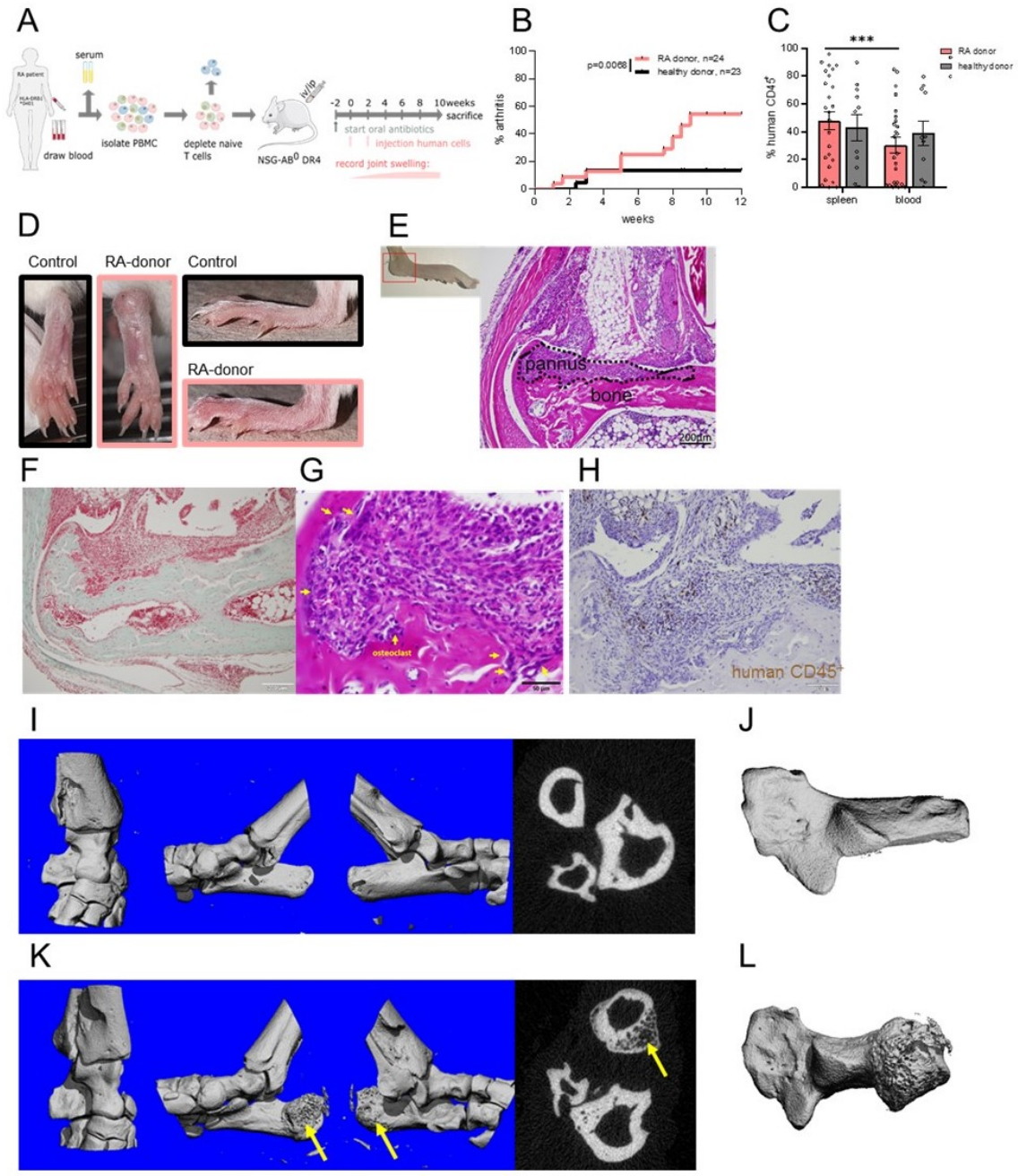

Figure 9 Cells of the peripheral blood of RA patients trigger an RA-like joint disease in NSG-DR4 mice A) Study protocol for the development of humanized mice with inflammatory joint disease triggered by the implanted human immune system. iv intravenous; ip intraperitoneal. B) Kaplan-Meier Curve of arthritis development comparing incidence of arthritis between mice engrafted with cells from healthy or RA donors. C) Efficiency of human engraftment by FACS staining of human CD45 in blood and spleen of humanized mice, separated by healthy and RA donor. Human engraftment within spleen and blood was compared using 2way Anova and Sidak post test. Data are presented as mean ± SEM. ***$p \leq 0.001$ D-G) Hallmarks of RA: D) clinical images representing inflammation by joint swelling and redness of joints, E) H&E staining demonstrating pathological changes including immune cells that infiltrate the tissue leading to an invasive and destructive pannus that leads to bone erosions (dashed line), F) Cartilage damage shown by safranin O staining, and G) increased osteoclastogenesis shown by H&E staining. H) Immunohistochemistry staining of human CD45 cells of an erosive joint. I-L) Micro computed tomography analysis of calcaneus

comparing the arthritis and control cohort. Joints were harvested ten weeks post-injection. I, J) Shows the control mouse injected with cells from a HLA-DR4 healthy donor and K, L) the calcaneus with osteophyte formation of an arthritis mouse which was sacrificed six weeks after the first clinical signs of arthritis.

We performed, immunohistochemistry staining of human $CD45^+$ on different organs including pancreas, salivary gland, kidney, heart, lung, and skin to confirm that the infiltration of human CD45 is, except for lung and spleen, joint specific (Figure 10). Regarding lung infiltration, we identified infiltrating human $CD45^+$ cells mostly around blood vessels (Figure 2B). Within skin tissue we did not see any human immune cells. Without depletion of naïve T cells prior injection, we saw mild skin infiltrations (data not shown)

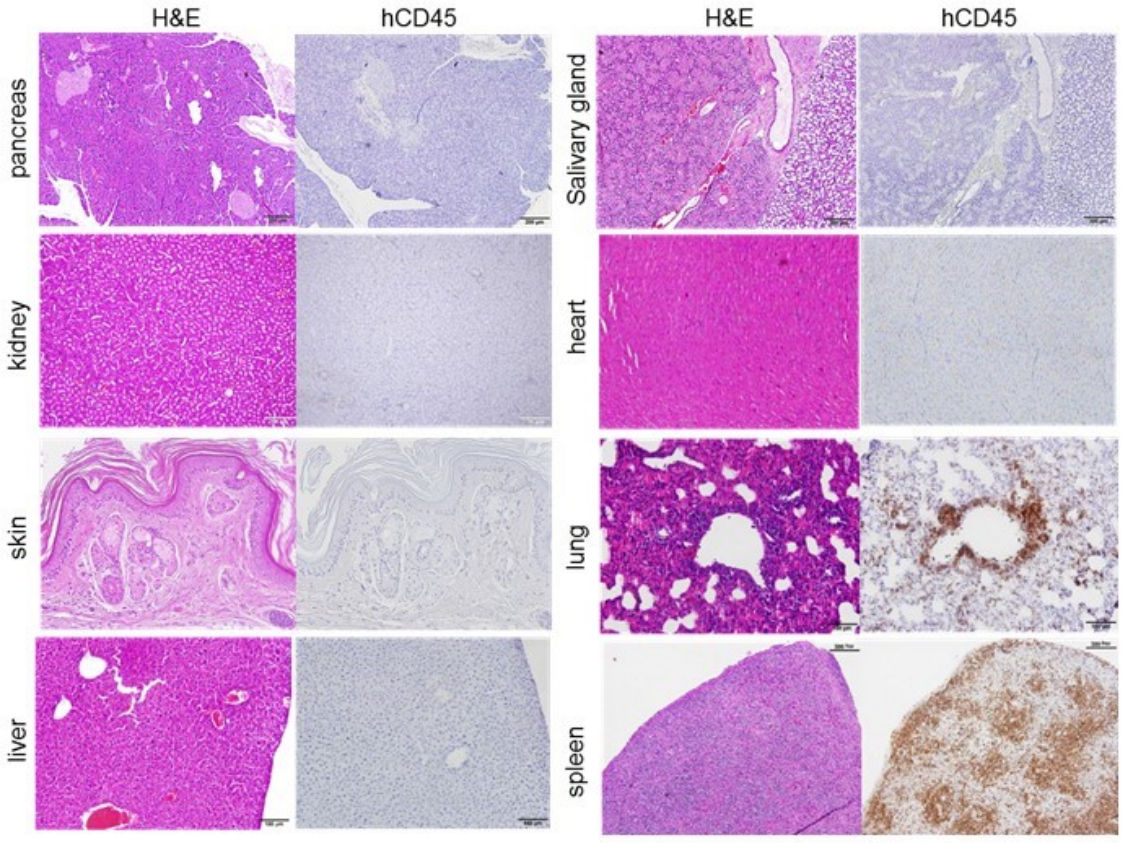

Figure 10 Tissue specific infiltration of human immune cells. Representative H&E and human CD45 immunohistochemistry stainings of different organs including pancreas, kidney, skin, liver, salivary gland, heart, lung, and spleen.

To identify the human cell composition we profiled the human immune cell content using flow cytometry. T cells represented the main human immune cell, whereas antigen presenting cells including B cells, monocytes, macrophages, and dendritic cells only occured in a minority of mice or decent amount (Figure 11A). As T cells dominate the human immune cell composition in this model, we performed detailed phenotyping of these cells. Compared to the corresponding donor, there is an increase in Th1 frequencies, which is even more pronounced in mice injected with cells form RA patients, and Th1 cells represent the dominant effector cell. Moreover, regulatory splenic T cells (Tregs) were decreased in these mice (Figure 11B). Beside this shift towards Th1 cells we also identified an increase in effector and effector memory T cells and hence antigen experienced cells, whereas central memory and naïve T cells were diminished (Figure 11C,D). The absence of naïve T cells is due to our study protocol as we depleted these cells prior injection.

Since Th1 cells represent the dominant effector cells in this mouse model, we performed another mouse experiment, where we aimed to enrich the Th1 engraftment. Therefore, we cultured $CD3^{+}CD45RA^{-}$ cells in Th1 polarizing media (media containing IL-12) and injected these cells seven to ten days after the first injection (Figure 11E). Preliminary data, revealed that boosting the Th1 cells via second injection increases the arthritis incidence in humanized NSG-DR4 mice.

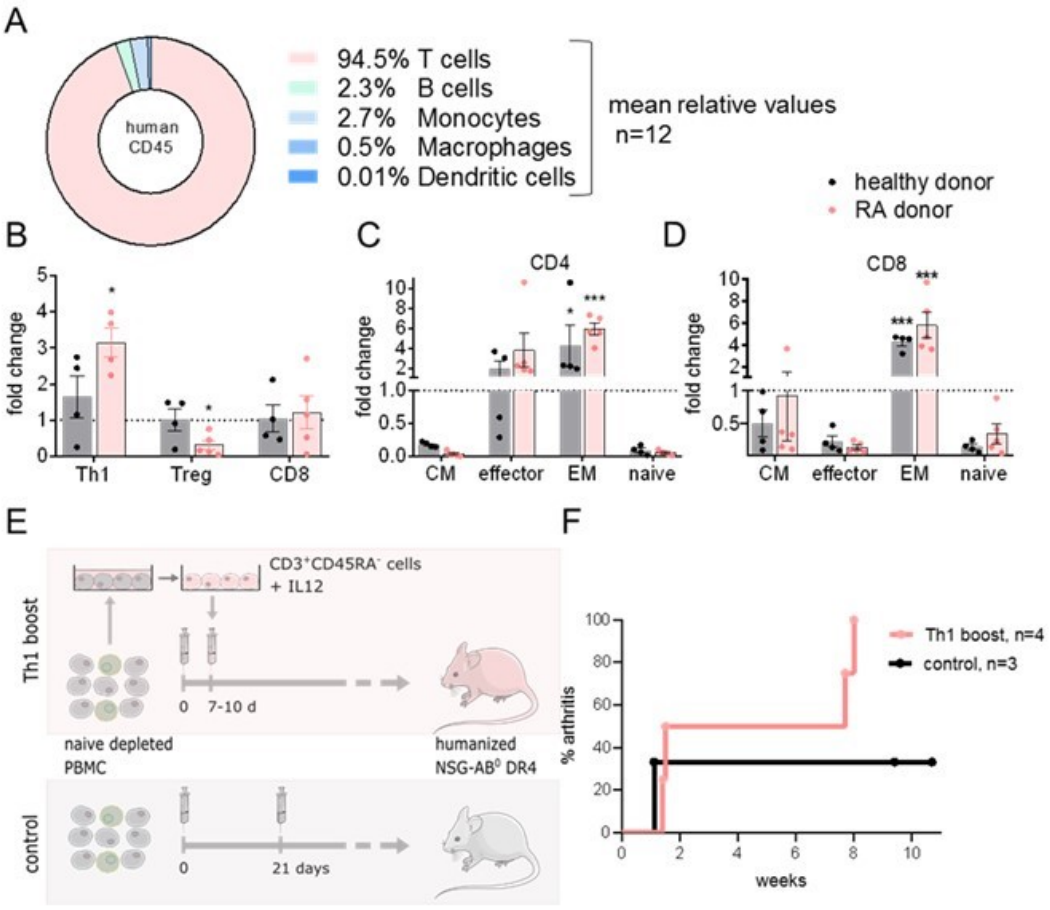

Figure 11 T cells especially Th1 cells dominate the human immune cell composition. A) Relative mean of human cell distribution in splenocytes of twelve humanized NSG-DR4 mice. B-D) Human T cell phenotyping in splenocytes normalized on corresponding donor to investigate if there is an increase or decrease in distinct cell populations of engrafted mice. Each data point represents one sample and data are presented as mean ± SEM. *$p \leq 0.05$ ***$p \leq 0.001$. E) Illustrates the study protocol for the Th1 boost experiment. Humanization was initiated by transferring PBMCs without naïve T cells of RA donors in two mice each. $CD3^{+}CD45RA^{-}$ cells were cultured for seven to ten days in Th1 polarizing media and consequently injected into the Th1 cohort. The control group was boosted with frozen naïve T cell depleted PBMCs of the same donor. F) Preliminary data comparing arthritis incidence between control and Th1 boosted mouse cohort.

Abatacept is a CTLA4-Ig fusion protein and used for the treatment of RA patients. It binds to CD80 and CD86 leading to inhibited T cell activation by blocking the costimulatory signal. Furthermore, it inhibits T cell proliferation *in vitro* (129,130). We tested whether this established RA drug affects arthritis development in these mice. Similar to the previous experiment, mice were injected with naïve T cell depleted PBMCs of RA donors. Additionally, we treated the mice prophylactically with abatacept or PBS (as control injection) two weeks post-injection (Figure 12A). Abatacept treatment, did not affect humanization efficiency, but intriguingly it totally prevented arthritis development (Figure 12B-F).

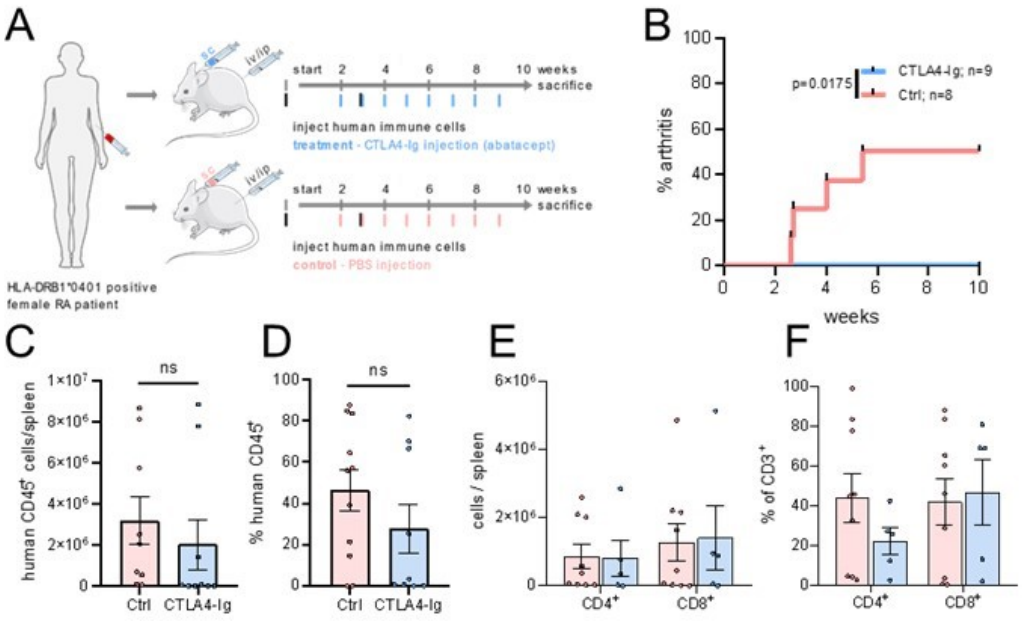

Figure 12 Prophylactic abtatacept (CTLA4-Ig) treatment prevents arthritis development in humanized mice. A) Experimental scheme. Engrafted NSG-DR4 mice were divided into two groups: abatacept treated (n=9) and untreated (PBS, n=8). Cell injections were performed intravenous and intraperitoneal and treatment was administered subcutaneously prior first signs of arthritis. B) Kaplan-Meier curve representing arthritis incidence and comparing treated and untreated humanized NSG-DR4 mice. C-F) Relative and absolute values of human CD45, CD4, and CD8 T splenocytes of engrafted NSG-DR4mice. Graph shows individual values with mean ± SEM

12.Discussion

This study reports the importance of Th1 cells in human diseases, more precisely COVID-19 and RA.

12.1. COVID-19

We phenotyped the T cell composition of patients infected with SARS-CoV-2. Early in the COVID-19 pandemic there was little information about the immune cell composition present in severe and critical SARS-CoV-2 infected individuals. Our study represented one of the most comprehensive analysis of T cell subsets of SARS-CoV-2 infected patients. We chose to use flow cytometry to profile the T cell content, based on surface receptors. By using this method we were able to quantitatively measure absolute and relative amount of distinct T cell subsets. Therefore, our study provides an in-depth analysis on the lymphocyte subsets. In line with other studies (131,132), we found decreased cell counts of B and T lymphocytes in the severe and critical cohort compared to healthy donors. However, we did not identify significant differences between the two patient groups. Li Tan *et al.* concluded from their data that decreased lymphocyte counts, might be an effective marker for COVID-19 severity and hospitalization (131). The mechanism for lymphopenia are not fully understood but might be explained by impaired lymphocyte apoptosis or proliferation (133). It has also been shown that T cell counts increase slowly with disease resolution and serum levels of pro-inflammatory cytokines are decreased compared to the illness period. Therefore, lymphopenia might be the result of high serum concentrations of pro-inflammatory cytokines that regulate proliferation and apoptosis of T cells negatively (48).

Based on the relative T cell distribution, we identified segregation of the healthy and COVID-19 patients. We did not identify clear cluster separation between the severe and critical patients, however interestingly, four of six patients that succumbed due to critical SARS-CoV-2 infection were assigned to a sub-cluster, which was characterized by low naïve $CD4^+$ T cell frequencies and therefore decreased T cell receptor reperoire. Garcia-Gasalla *et al.* compared severe or critical and recovered patients and identified significantly elevated relative naïve $CD4^+$ T cell numbers in the severe/critical patient cohort (134). In another study, naïve $CD8^+$ T cells were significantly lower in the patients

cohort compared to healthy controls, whereas they did not identify a significant reduction in the relative naïve $CD4^+$ T cells (only the absolute numbers were significantly lower) (42). However, only five of 39 included patients succumbed due to COVID-19 and therefore might be underrepresented in their study. In addition, we identified expansion of $CD8^+CD28^-$ cells. Loss of CD28 expression on cytotoxic T cells is a well known immune response of HIV-infected persons (135).

Furthermore, we measured a decrease in relative Th1 cells in critical cases and an increase in Th17 in both severe and critical patients. The shift towards Th17 was also reported by De Biasi *et al.* (42) and viral clearance is associated with a Th1 phenotype (133). However, it has also been reported that patients with severe virus infections have a prominent Th2 response (42,136). Within our patient cohort we could not identify an increase in Th2 cells compared to healthy controls. The data on Treg levels are controversial in COVID-19, Tan *et al.* reported increased Treg levels in these patients (137), while other studies found a decrease in Treg expression (37,39) . In our patient cohort we did not identify any changes in the Treg population. This might be based the time of the blood draw, a case report of an asymptomatic SARS-CoV-2 infected male subject showed that the Treg levels were similar to healthy controls on day seven, increased gradually since then, peaked at day 22, and decreased slightly at day 28 (138). We believe that these changes in T helper subsets result as consequence of interleukin 6 (IL-6) upregulation.

CRS is associated with a massive release of several pro-inflammatory cytokines and especially IL-6 has a key role in the pathogenesis of this life-threatening syndrome (36,37,127). Briefly, IL-6 can shift the Th1/Th2 balance towards the Th2 phenotype (139), may induce the differentiation into Th17 cells, and inhibit the induction of Tregs (140). However, in our study only Th1 cells are decreased while Th2 cells are unaffected. Hence, other mechanisms might be responsible for the Th1 loss. As viral persistence might result due to impaired T cell function, we examined the proliferation and activation of these T cells. $CD4^+$, especially Th1, and $CD8^+$ cells co-expressing the proliferation marker Ki67 and activation marker CD38, were significantly elevated in severe and critical COVID-19 patients. This data indicate that proliferation and activation of T cells, in particular the anti-viral T cell subsets are induced during the acute phase of COVID-19 infection. This goes in line with what has been reported in other viral infections (141,142), where they also described that after viral clearance these proliferating T cells

are diminished while the expressing of activation markers can be long lasting and correlates with a longer viral persistence in the blood (141). In a SARS-CoV-2 case report of an asymptomatic patient they also identified increased Ki67 expression that peaked during the acute phase of infection, gradually decreased with viral clearance, and was similar to healthy controls on day 28 (138).

In line with previous reports (37), we have also measured increased plasma IL-6 concentrations in our patient cohort, which were associated with severe COVID-19 courses. Furthermore, it has been shown that IL-6 levels are also associated with death, as it is elevated in non-survivors during the acute-phase of a SARS-CoV-2 infection (143), correlates with respiratory failure and CRP levels (144), and can therefore be used as a predictor of outcome and to monitor COVID-19 patients. Is has been shown that 80pg/mL might be the optimal IL-6 cutoff to predict respiratory failure in COVID-19 patients (145). By using this IL-6 cutoff we were able to show that elevated IL-6 values negatively predict the Th1 $Ki67^{+}CD38^{+}$ and $CD8^{+}$ $Ki67^{+}CD38^{+}$ content as well as total $CD8^{+}$ T cells. Furthermore, if we include all IL-6 values, we also saw a negative correlation with IL-6 and CD3 cell counts, and $Ki67^{+}CD38^{+}$ $CD4^{+}$ T cells, more precisely Th1 $Ki67^{+}CD38^{+}$ T helper cells. We suggest that these inverse correlations of IL-6 and the antiviral subsets are the primary cause of virus persistence. We suggest that the decrease in activated and proliferating Th1 and $CD8^{+}$ T cells, implicated in host antiviral defense, might be the result of cellular exhaustion reported in COVID-19 (42,146). T cell exhaustion has been suggested to predict a severe course of disease in COVID-19 (41,42). Alternatively, cytokines produced during CRS could directly impact T cell proliferation or viability. Therefore, we tested whether increasing IL-6 concentrations could inhibit anti-CD3/CD28 induced T cell proliferation. As no differences were detected in the *in vitro* experiments, IL-6 may not be directly responsible for reduced T cell activation and proliferation in CRS. However, other cytokines present in CRS may impact T cell activation and proliferation. Diao *et al.* reported that levels of $CD4^{+}$ and $CD8^{+}$ T cells correlated negatively with different cytokines, including IL-6. Therefore, they suggested that IL-6 together with TNF-α, and IL-10 may reduce T lymphocyte counts by impairment of T cell proliferation and survival (48). In summary, we suggest that severe CRS is associated with impaired antiviral T cell activation and may thereby limit SARS CoV-2 clearance. Moreover, we also correlated clinically relevant parameters, including viral load, CRP, ferritin, oxygen supplementation, and duration of symptoms with frequencies of the described T cell

populations. Beside a positive correlation between central memory $CD8^+$ T cells and viral loads, we did not identify any correlations between T cell populations and the mentioned clinical parameters.

Early in the pandemic, little was known about potential therapeutic medication of severe/critical COVID-19. Our clinical data of IL-6 blockade allowed us to suggest that blockade of IL-6 signaling by tocilizumab may indirectly affect other cytokines and chemokines implicated in lymphopenia. In rheumatoid arthritis, IL-2, TNF-α and VEGF serum levels decreased following tocilizumab treatment (147). Of note, TNF-α causes apoptosis of T cells at higher concentrations (148) thus suggesting involvement in the development of lymphopenia. Our results suggest that IL-6 blockade leads to decreased inflammation and improvement of lymphopenia. In addition, it may allow reactivation of the antiviral Th1 cell defense in severe CRS. The data on tocilizumab in COVID-19 are quite conflicting, it has been shown that tocilizumab does not affect mortality but reduces the risk of mechanical ventilation (149). In another study researchers were able to show that tocilizumab treated patients were not only less likely to require invasive mechanical ventilation, but also showed lower mortality rates. This was independent of administration (intravenously or subcutaneously) (150). However, Stone *et al.* reported in a randomized double-blinded placebo-controlled trial that early tocilizumab treatment did not prevent intubation or death in COVID-19 hospitalized patients by disrupting the cytokine storm present in these patients. Their patient cohort was ethnically diverse and had not been intubated before (151). Nevertheless, in April 2022 the WHO recommended the use of tocilizumab for severe and critical COVID-19 patients based on the promising results of the largest clinical trial (RECOVERY) (152).

We are well aware of the preliminary nature of these findings limited by a small sample size. Thus, a larger sample size and longer time courses are necessary to evaluate significant changes within these cell subsets. However; many of our findings have been confirmed by other articles published later in the pandemic.

In conclusion, the T cell response to SARS CoV-2 infection is characterized by activated and proliferating Th1 and $CD8^+$ cells. In the situation of CRS these cells are diminished, which may impair viral clearance. Blockade of excessive IL-6 release reverses these changes in some patients indicating a need for larger studies to proof the efficiency of anti-IL6 therapy on Th1 and $CD8^+$ T cells in COVID-19.

12.2. Rheumatoid arthritis

In our second study, we investigated the role of Th1 cells in an autoimmune disease, more precisely RA, by transferring naïve T cell depleted PBMCs of RA patients to immunodeficient NSG-DR4 mice. Th1 cells represent the main human immune cell subset in these mice. Therefore, we suggest that these immune cells are crucial in initiating an RA-like disease in this model. This data goes in line with the literature, as Th1 cells belong together with Th17 and Tregs to the key subsets in RA (94,153).

Our work focuses on a new humanized mouse model, which in general belong to the most promising strategies in basic research to analyze the human immune system *in vivo* without putting the patients at risk. Our data describes a novel mouse model and how to transfer immune cells, of the peripheral blood of RA patients, to initiate a patient specific inflammatory joint disease and therefore study the pathogenesis of RA *in vivo* on a human immune system.

To study RA there are plenty of mouse models available, each addresses specific aspects of the disease. However, due to differences in the adaptive and innate immune system of human and mouse, these conventional models are only partially suited to study e.g. novel therapies. Therefore, a mouse model that accurately mirrors the autoimmune processes is needed. We were able to develop a humanized mouse model to study the pathogenesis of RA. After injecting PBMC of RA patients these mice develop an RA-like erosive joint disease that is driven by the implanted human immune system without prior conditioning regimes, such as sub-lethal irradiation or chemotherapeutic agents (154,155). These mice show clinical signs of RA including joint swelling and reduced grip strength as well as pathological hallmarks including immune cell infiltration, pannus formation, increased osteoclastogenesis, cartilage damage and bone erosions. Furthermore, if we do not sacrifice mice at peak of disease but wait a couple of weeks, we see bone remodelling, more precisely formation of osteophytes. This may occur as a result of prior erosions in the area. In RA patients it has been shown that healing processes occur after inflammation has discontinued for several months, leading to formation of osteophytes (156). This has also been shown in RA patients after remission (157) and goes in line with our clinical observations in this mouse model. If we sacrifice the humanized mice shortly after peak of disease (active swollen joint), we did not observe any forms of healing. Here we suggest that earlier time points are preferable for future experiments, to evade bone repair mechanisms.

To date there is only one humanized mouse model of RA available that mimics some aspects of the disease. Weyand and co-workers developed a model of synovitis by implanting pieces of inflamed synovial tissues of RA patients subcutaneously into a pocket on the upper dorsal midline of immunodeficient NSG mice. In addition, they engraft the mice with PBMC of the same donor leading to complete tissue engraftment within one week. With this model the Weyand group was also able to reveal that Th1 cells drive the expression of pro-inflammatory cytokines and tissue-degrading enzymes (81). Besides implanting tissue (synovial tissue but also human thymus or fetal liver) there are also other strategies for the generation of humanized mice including introduction of human transgenes, or transfer of hematopoietic stem and progenitor cells. However, each of these methods have limitations. For tissue implantation, surgery is needed, which might be complex and samples are not easily available. The implantation of human transgenes will give researchers the opportunity to study disease relevant genes *in vivo*, but the observed immune responses will still be driven by mouse immune cells and there are distinct differences between human and mouse immune cell distribution, development, and regulation. The stem cell engraftment will lead to good engraftment rates of multiple lineages but the human T cells will still be restricted to the murine major histocompatibility complex (MHC) (114). Therefore, we decided to combine two of these methods to overcome some of these limitations. More precisely, we combined PBMC injections and transgenic expression of the RA risk allele HLA-DRB1*0401 to avoid GvHD and trigger autoimmunity. Blood, spleen, bone marrow, joints, and different organs of humanized mice were obtained and processed immediately after harvesting. The highest engraftment was found within the spleen and blood, whereas in the bone marrow only about 5% of human immune cells were detected (data not shown). This goes in line with other PBMC mouse models. In a humanized mouse model of type 1 diabetes they identified high human engraftment rates that plateaued within three to four weeks. They had high variability in human $CD45^+$ engraftment but overall they measured higher engraftment within the spleen than peripheral blood (median spleen around 60% vs blood 20-40% human $CD45^+$ cells, depending on intraperitoneal or intravenous injection) (158). In addition, in our model we see infiltration of human $CD45^+$ cells within joint (including sites of bone erosion) and lung, whereas kidney, liver, pancreas, salivary gland, heart, and skin are not affected. Notably, lung disease is common in patients with RA. Bronchial biopsies of patients with early-untreated RA and without clinical signs of lung involvement revealed that lymphocyte infiltration was more likely

in ACPA-positive patients compared to ACPA-negative and healthy controls. (159). Willis *et al.* reported that RA-related autoantibodies can be found in the sputum of subjects with early RA and subjects at risk of RA. Intriguingly, in a subset of the at-risk cohort, autoantibodies were not found in the serum. Therefore, they suggest that the lung might be a site of autoantibody production in early RA (160). Both studies underline the important role of the lung in the development of RA. However,the mechanisms that lead to the pathological changes of the lung and especially joints are currently unknown. One possible solution to answer this question, could be to investigate the immunologic differences present in the different mouse cohorts in future studies. We propose that single cell RNA sequencing, metabolomics and proteomics data of the synovial tissue of these mice will lead to a better understanding of the pathogenic mechanism that lead to arthritis in this model but also in the patients. Furthermore, it will also give us a broader knowledge of the cytokine milieu within the joint.

Development of GvHD is one of the major obstacles in the establishment of a humanized mouse model. However, by using NSG mice that carry the human HLA-DR4, depletion of naïve T cells prior injection, and identification of optimal cell number for the injection, we were able to completely avoid lethal or acute GvHD. More precisely, by naive T cell depletion, we are able to increase the window for these kind of experiments from about four to five weeks up to 16 weeks without lethal GvHD. Therefore, it gives us the opportunity to get new and exciting insights into the pathogenesis of RA, perform long-term effects on a human immune system *in vivo*, and identify and test novel therapies *in vivo* on a human immune system without putting patients at risk.

In previous experiments of our lab we were able to show that higher cell numbers result in increased numbers of human $CD45^+$ cells, but simultaneously increase the occurrence of GvHD. Due to this limitation, ten to fifteen million naive T cell depleted PBMCs seem to be the optimal amount for good engraftment without GvHD. Although in some cases, even lower numbers (2.5 - 4 x 10^6 cells) engrafted well (data not shown). Hence, specific cell ratios and or clones might be important to establish a functional human immune system in these mice. However, due to the optimization of our study protocol as described above, we were able to avoid GvHD and in addition increase the timespan for this kind of experiments from four to five weeks up to 16 weeks.

We are able get engraftment rates of up to 90% (mean 50%) human $CD45^+$ cells. However, we also see high variability in humanization. This might rely on one of the

following reasons: I) leakiness of the mouse mutations more precisely spontaneous development of mouse B, T, NK, and innate immune cells, whereby stable human engraftment is hindered (161). One potential solution to overcome this issue could be the exclusion of these mice. Therefore, one could measure the relative amount of these murine cells and/or immunoglobulin levels in the peripheral blood of NSG mice prior injection. II) Each patient is different and for some reasons there might be patients whose cells do not engraft well in NSG mice.

Mice humanized with cells from RA donors are more likely to develop an RA-like disease and prophylactic treatment with abatacept prevented disease initiation. However, 10% of the healthy cohort developed arthritis. Therefore, we have to identify the factors that lead to the initiation of the disease as well as examine why some of the healthy cohort developed the disease. Of note, we do not have any details about the medical history of some of the healthy subjects. Therefore, these age-matched controls might already have pre-clinical RA symptoms (e.g. autoantibody production).

Arthritis development is more pronounced in the ankle joints. This goes in line with what has been described in a spontaneous mouse model of RA. Holmdahl *et al.* revealed that in DBA/1 mice, which develop an RA-like disease spontaneously, arthritis development was restricted to hind paws and also more pronounced in ankle joints. In contrast, in CIA mice both front and hind paws are affected equally and arthritis occurs more often in the smaller joints (162).

T cells represent the majority of engrafted human immune cells, whereas B cell engraftment occur only in a minority of mice or decent amount. Sequential transfer (second injection three weeks after first injection) slightly improved the B cell engraftment. In another experiment we injected whole PBMC fraction of healthy subjects into NSG mice expressing human cytokines (NSG-SGM3). These mice showed increased levels of $CD19^+$ cells (mostly plasmablasts) when sacrificed four weeks after humanization (data not shown). However, they also developed clinical signs of GvHD. Of note, in this model arthritis development is independent of B cell engraftment. Therefore, T cells seem to be the crucial cell type regarding initiation of an RA-like disease. Results of another study support this hypothesis, by using an antigen-induced mouse model. The study concluded that $CD4^+$ T cells, but not $CD8^+$ T cells or B cells, are the crucial cell type in the initiation of arthritis (82). To identify the dominant human T cell subsets present in this mouse model, we comprehensively profiled the T cell

content and function in splenocytes of engrafted mice. Furthermore, as we were also interested if the immune cell composition is changed following engraftment, we measured the same FACS panel directly after blood draw of the donors (baseline). Th1 cells ($CXCR3^+CCR6^-$) represent the main T-helper cells in this mouse model. In line with this, Th1 cells are the predominant effector cells in joints of RA patients (85), and belong, together with Th17 and Tregs, to the key T cell subsets in joint inflammation of RA patients (94,153). Furthermore, measuring the blood of RA patients revealed that 40% of the citrulline-reactive CD4 T cells were CXCR3 positive (163). Expansion of the human immune cells in the recipient mouse, suggested significant proliferation of the injected cells. By flow cytometry, we were able to determine an increased proliferative potential (Ki67 expression) of human $CD4^+$, including Th1, and $CD8^+$ splenic T cells following engraftment.

Prophylactic abatacept treatment prevented the initiation of an RA-like disease. Abatacept treatment did not affect human engraftment. More precisely, relative and absolute number of human CD45, human CD4, and human CD8 T cells were not affected following treatment. Furthermore, we hypothesized that treatment might reduce proliferation of T cells. However, Ki67 expression did not differ between treated and untreated group (data not shown).

One potential limitation of our study might be the small sample size. Furthermore, we see high variability in human engraftment but also arthritis incidence between our individual experiments/mice. Initiation of arthritis might be dependent on other environmental factors. In CIA rats and mice it has been shown that disease severity is not only highly variable but also dependent on environmental factors or behavior-dependent including for example stress. They speculate that due to stress or fighting small skin lesions occur, which lead to inflammation or chronic immune responses to pathogens or antigens that finally trigger arthritis (162). In our model we also see severe joint swelling after small skin lesions of the ankle joint. So far arthritis development or bone erosions are dependent on a functional human immune system, although joint swelling of the tendons can also occur due to skin lesions prior injection. Taken together, we are able to transfer the disease of HLA-DR4 positive RA patients to NSG-AB^0 DR4 mice just by intravenous and intraperitoneal injections of PBMCs. However, currently we do not fully understand the mechanisms that lead to the initiation and why some of the mice humanized with cells of RA donors did not develop an RA-like disease.

Another question that is remaining and that we want to tackle in a following study is the identification of theTCR repertoire and autoantigens using fresh tissue. We hypothesize that specific autoreactive T cell clones trigger arthritis in NSG-DR4 mice. Identification of these TCR sequences and their target antigens is of great interest as the TCR repertoire is central to the immune system function. The TCR is responsible for the recognition of antigens and TCRs drive antigen specific responses by recognizing complexes consisting of an antigen and the MHC. The identification of arthritogenic T cell clones will be crucial to understand the mechanisms that are responsible for the initiation of the disease. Moreover, detecting the TCR repertoire of arthritogenic T cells will be an important step towards the identification of antigens that trigger or initiate RA. We hypothesize that these cells will be found in the inflamed synovium of humanized mice. Consequently, we could screen for these relevant TCR sequences in the corresponding frozen patient sample and subsequently establish T cell clones or even an arthritogenic T cell line that can be again transferred into NSG mice. Hence, we could examine the effects of these T cell clones on the arthritis development and disease severity and also look for possibilities how to expand or diminish these clones, and thereby modulate the disease activity of these mice. By TCR sequencing of arthritogenic T cells found in the joints of our mice we could also identify antigens driving the T cell responses. Different research groups reported (164–166) distinct autoantigens in RA, however it is uncertain if these antigens really trigger the disease. Our novel mouse model would help us to identify arthritogenic antigens and evaluate the T cell clonality in our mouse model. Furthermore, this information should also help to guide the design of new therapeutic clinical trials. Furthermore, we plan to get a better understanding of the immune cell interactions that lead to RA. We suggest that injecting distinct cell populations of the PBMC fraction will help us to tackle this question. Besides studying the reactivity of specific cell subsets, we also aim to assess the microbiome involvement. We and others (165,167), suggest that imbalance of the microbiome is associated with RA development as it could lead to chronic inflammation. Consequently, we could improve our understanding of the gut-joint axis and how it affects metabolism. Furthermore, we could investigate if there is a link between the microbiome and arthritis severity or prognosis. As these experiments will be performed on a humanized mouse model with functional human immune system, we can easily transfer the outcome to the patients.

Humanized mice belong to the most promising strategies in basic research to analyze the human immune system *in vivo* without putting the patients at risk. Our data describes a

novel humanized mouse model and how to transfer immune cells, predominantly Th1, of the peripheral blood of RA patients, to initiate a patient specific inflammatory joint disease and therefore study the pathogenesis of RA *in vivo* on a human immune system. As described above, there are distinct limitations and open questions remaining in this model. Therefore, further studies are needed. Although we believe that the current data shows that these mice mirror the autoimmune processes of RA patients leading to an RA-like disease that is dependent on the implanted human immune system.

13.References

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