DIPLOMARBEIT

In vitro Induced Regulatory T-Cells can Reduce Severity of Lupus Arthritis

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Ehrenwörtliche Erklärung

Ich erkläre hiermit, die vorliegende Arbeit selbst verfasst zu haben. Außer den angeführten Hilfsmitteln und Quellen wurden keine anderen verwendet. Stellen, die ich wörtlich oder sinngemäß aus anderen Arbeiten übernommen habe, sind als solche gekennzeichnet.

Barbara Schwarzecker, eh.

Abstract

Introduction. Arthritis is a common complaint in human systemic lupus erythematosus (SLE), but is rarely seen in lupus models. Pristane-induced lupus (PIL) is an established model for SLE and is associated with lupus-arthritis when induced in BALB/c mice. Regulatory T-cells (Treg) are essential for maintaining peripheral tolerance and, in SLE, their numbers and suppressive capacity inversely correlate with disease activity. These findings indicate an important step in the breakdown of self-tolerance and the development of the autoimmune response in SLE. We herein investigate if *in vitro* induced Treg (iTreg) can affect typical features of PIL arthritis.

Methods. BALB/c mice were injected i.p. with either 0.5ml of pristane (PIL-group) or PBS as control and killed after 8 months. Naive $CD4^+$ thymocytes were sorted and cultured under Treg-inducing conditions (including TGFβ and IL-2). Cell suspensions with >80% of $CD4^+FoxP3^+$ cells (iTreg) were injected intravenously: a) once when PIL was induced ($5x10^6$ Treg; Treg-boost-group) or b) every 4 weeks ($1x10^6$, Treg-repeated group). Animals were monitored for clinical signs of arthritis (paw swelling, grip strength) and at the end of the experiment hind paws were analyzed; using an image analysis system (Osteomeasure®) to assess and compare disease severity, the following features were evaluated: extent of inflammation, extent of bone erosion, number of osteoclasts and cartilage degradation. To estimate and compare disease severity with only one parameter, the arthritis severity score (ASS) was calculated.

Results. Clinically, the PIL-group was affected the most: it showed the earliest onset of symptoms (week 14) with the most severe course over 8 months. The monthly injection of $1x10^6$ Treg resulted in a significantly milder course seen in a higher mean grip strength (2.964 ± 0.024 vs. 2.732 ± 0.063, $p<0.01$) and less mean paw swelling (0.044 ± 0.020 vs. 0.360 ± 0.069 , $p < 0.01$) compared to the PIL-group. In histological analysis, we found 62% of PIL-mice and only 33% of Treg-rep mice to have erosive arthritis. The monthly injection of Treg significantly reduced all histological parameters (inflammatory area 0.188 ± 0.0574 vs. 0.688 ± 0.113, $p<0.001$; erosive area 0.011 ± 0.009 vs. 0.069 ± 0.017, $p<0.01$; number of osteoclasts 2.000 ± 1.125 vs. 9.143 ± 1.999, $p<0.01$; cartilage degradation 0.059 ± 0.004 vs. 0.187 ± 0.033, $p<0.01$). The single Treg boost of $5x10^6$ could not prevent joint manifestation, but seemed to have a retarding effect indicated by a slight retardation in 'loss of grip strength' and in a significantly less erosive area compared to PIL (0.023 ± 0.006 vs. 0.069 ± 0.017, $p<0.01$).

Conclusion. Repeated injections of *in vitro* induced regulatory T-cells can ameliorate the clinical and histological severity of pristane-induced arthritis. A single boost of Treg at the time of disease induction does not prevent joint manifestatin, but appears to have a retarding effect in disease progression.

Zusammenfassung

Hintergrund. Arthritis ist ein häufiges Beschwerdebild im Rahmen des humanen Sytemischen Lupus erythematodes (SLE), wird aber kaum in Lupus-Modellen gesehen. Das Tiermodell des Pristan-induzierten Lupus (PIL) ist ein etabliertes Modell in der SLE-Forschung und macht es anhand der Tatsache, dass es in BALB/c Mäusen Arthritis induziert, zu einem wertvollen Instrument deren Erforschung. Regulatorische T-Zellen (Treg) sind essentiell in der Immunhomöostase und Erhaltung der peripheren Toleranz. Im SLE korreliert die Anzahl und das immun-modulatorische Potential dieser Zellen invers mit der Krankheitsaktivität. Diese Erkenntnis weist darauf hin, dass eine verminderte Anzahl von Treg wesentlich zur Entstehung von Autoimmunerkrankungen beiträgt. Ziel dieser Studie ist es, zu beurteilen, in wie weit *in vitro* induzierte Treg (iTreg) den Krankheitsverlauf der PIL-Arthritis beeinflussen können.

Methodik. 6-8 Wochen alte weibliche BALB/c Mäuse erhielten eine intraperitoneale Injektion von entweder 0.5ml Pristan (PIL-Gruppe) oder PBS (Kontroll-Gruppe). Naive $CD4^+$ Thymozyten wurden aus Thymi junger Versuchsmäuse isoliert und im Treg-induzierenden Medium, welches TGFß und IL-2 enthielt, kultiviert. Zell-Suspensionen mit >80% $CD4^+Foxp3^+$ Zellen (iTreg) wurden anschließend intravenös injiziert: a) einmalig zum Zeitpunkt der PIL-Induktion ($5x10^6$ Zellen; Treg-boost Gruppe) oder b) alle 4 Wochen ($1x10^6$ Zellen; Treg-repeated Gruppe). Die Versuchstiere wurden über 8 Monate auf klinische Zeichen der Arthritis beurteilt (Pfotenschwellung und Griffstärke) und am Ende der Beobachtungszeit wurden die Hinterpfoten mit Hilfe von Osteomeasure® histologisch ausgewertet. Folgende Paramter wurden hierfür zur Beurteilung des Ausmaßes der Gelenksbeteiligung verwendet: Ausmaß der Entzündungsfläche, Ausmaß der Knochenerosion, Anzahl von Osteoklasten, Verlust von Knorpelfläche. Zum Abschätzen der Stärke der Gelenksbeteiligung mit einem einzelnen Parameter wurde der 'Arthritis severity score' (ASS) berechnet.

Ergebnisse. Im klinischen Verlauf war die PIL-Gruppe am stärksten betroffen: sie zeigte das früheste Auftreten von Symptomen, sowie den schwersten Krankheitsverlauf über 8 Monate. Die monatliche Injektion von 10^6 Treg führte zu einem milderen Verlauf mit einer signifikant höheren durchschnittlichen Griffstärke (2.964 ± 0.024 vs. 2.732 ± 0.063, $p<0.01$) und verminderten durchschnittlichen Pfotenschwellung (0.044 ± 0.020 vs. 0.360 ± 0.069 , $p < 0.01$). Die histologische Auswertung zeigte, dass 62% der Mäuse der PIL-Gruppe und nur 33% von der Treg-rep Gruppe eine erosive Arthritis aufwiesen. Die monatliche Injektion von Treg konnte zusätzlich alle histologischen Parameter signifikant vermindern (Außmaß der Entzündung 0.188 ± 0.0574 vs. 0.688 ± 0.113, $p<0.001$; Ausmaß der Erosion 0.011 ± 0.009 vs. 0.069 ± 0.017, $p<0.01$; Anzahl der Osteoklasten 2.000 ± 1.125 vs. 9.143 ± 1.999, $p<0.01$; Verlust der Knorpelfläche 0.059 ± 0.004 vs. 0.187 ± 0.033, $p<0.01$). Die einmalige Gabe von $5x10^6$ Treg konnte die Gelenksbeteiligung nicht verhindern, konnte jedoch die Krankheitsprogression verzögern: Klinisch äußerte sich dies in einem späteren Auftreten des Verlustes der Griffstärke und histologisch in einer signifikant verminderten erosiven Fläche im Vergleich zu PIL-Mäusen (0.023 ± 0.006 vs. 0.069 ± 0.017, $p<0.01$).

Schlussfolgerungen. Wiederholte Injektionen von $1x10^6$ *in vitro* induzierten regulatorischen T-Zellen mildern sowohl den klinischen, als auch den histologischen Verlauf der Pristan-induzierten Arthritis. Eine einmalige hochdosierte Injektion von $5x10^6$ Treg zum Zeitpunkt

der Krankheitsinduktion kann die Entstehung der Krankheit nicht verhindern, jedoch die Krankheitsprogression verzögern.

1 Introduction

1.1 Systemic lupus erythematosus

1.1.1 History

The description of a lupus-like disease can be tracked back to the ancient Greek physician Hippocrates, who first described a red facial rash combined with severe cutaneous ulcerations. The 13th century phyisician Rogerius attributed the term lupus (*lat.* wolf) due to the fact that these facial lesions, which are often seen in lupus patients, reminded him of a „wolf's bite". Thereupon the myth arose that patients suffering from this disease will turn into werewolves and thus were feared and excluded by society. In 1851 the French dermatologist Cazenave determined the term "lupus erythematosus" describing it as "a rare condition, which appears most frequently in young females who are otherwise healthy, attacking the face chiefly". Until then it was not known, that lupus also has a systemic component.
The 19th century Viennese dermatologist Moriz Kaposi, however, first described the systemic signs of lupus, which include fever, weight loss, lymphadenopathy, anemia and arthritis. He also defined that lupus erythematosus had no relation to the cutaneous form of tuberculosis, called lupus vulgaris. In the following decades researches associated more and more symptoms of lupus patients with the disease: Baehr described the involvement of the kidney ("lupus nephritis"), and Libman and Sacks indentified endocarditis as a symptom of systemic lupus erythematosus (SLE). In the late 1950´s George Friou introduced the immunofluorencent technique to detect antinuclear antibodies (ANAs), which became a remarkable achievement in the understanding of the pathogenesis of SLE and other autoimmune diseases.[1,2,3]

Even though major efforts and achievements in understanding the complexity of SLE were made since its first clinical description, many questions still remain unanswered and will be subject of further investigations.

1.1.2 Pathogenesis

Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disease of which the underlying etiology is largely unknown. Genetic, epigenetic, environmental and hormonal factors seem to cause immunological dysregulation, leading to autoreactive B- and T-cells, production of autoantibodies and immune complex formation. On the following pages possible influences on the development of SLE will be discussed.

1.1.2.1 The influence of genetics

Previous epidemiologic studies reflect a strong genetic component to the pathogenesis of SLE, which can be exemplified in the following data: Close relatives of affected patients are at higher risk of developing SLE; the prevalence of SLE for female first-degree relatives is estimated to be 2.64 per 100 SLE patients in contrast to a prevalence of 0.4 per 100 normal controls.[4] Twin studies illustrate that monozygotic twins show a higher concordance of disease than dizygotic twins (24% vs. 2%).[5] Furthermore, the probability to develop another autoimmune disease is higher among first-degree relatives.[6]

Due to these and many other epidemiologic studies investigation currently focuses on the role of genetics in the development of autoimmune disorders. Advanced techniques in gene sequencing have already led to the identification of over 40 susceptibility gene loci associated with SLE development. Many of these loci are situated within or near gene-encoding proteins, which have a relevant function in specific immune pathways. Aberrations from these pathways lead to an altered clearance of immune complexes, and defective B-/T-cell function and signaling. For instance, one protein important to T-cell functioning is PTPN22 (protein tyrosine phosphatase 22), which regulates T-cell-receptor (TCR) signaling. A single nucleotide polymorphism (substitution of arginine with tryptophane) results in an upregulation of TCR signaling and the presence of lupus-like autoantibodies. [7]

1.1.2.2 The influence of epigenetics

Epigenetic mechanisms such as DNA methylation and histone modification have been studied in patients with SLE. Previous studies claim that patients suffering from SLE exhibit reduced DNA methylation.[8] This hypomethylation leads to an overtranscription of genes. A study of Mi *et al* showed that the promoters of IL-4 and IL-6 genes were hypomethylated in lupus T-

cells. This led to an increase of IL-4 and IL-6 (B-cell differentiation factors) which correlated with the severity of disease manifestation.[9]

Histone modification is another important epigenetic mechanism. In general, hypoacetylation of histones leads to a higher transcription rate. Patients with active SLE showed a reduced expression of histone deacetylases (HDACs) such as HDAC2 and HDAC7, leading to histone hypoacetlyation.[10]

Unlike inherent and permanent genetic changes, epigenetic alterations are reversible and may be influenced by various environmental factors.

1.1.2.3 The influence of the environment

It is well known that the environment influences the incidence and the course of SLE. UV-light for example, is known as one of the triggers for SLE. UVA and UVB not only can cause skin lesions, but also trigger flares of the systemic manifestation. Interestingly, a vitamin D-deficiency, which can be caused by persistent abstention from sun exposure, can also lead to an impaired function of the immune system with a loss of tolerance.[11] Yet the correlation between levels of vitamin D and disease activity remains controversial.[12,13,14]

Smoking has been linked to various autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and SLE. The toxic compounds in cigarettes (e.g. tar, nicotine, polycyclic aromatic hydrocarbons, etc.) contain high concentrations of free radicals, which interact with DNA, and hence, can cause genetic aberration and gene activation. Furthermore cigarette smoke has a modulating effect on pro-inflammatory mediators including IL-6.[15]

The exposure to certain viral infections is also linked to SLE development. Here the Epstein-Bar virus (EBV), cytomegalievirus (CMV), and parvovirus B19, have to be mentioned. EBV has a high prevalence in the population and thus it is hard to prove a definite association to SLE; studies, however, showed that all patients developed antibodies to the EBV protein EBNA-1 before developing anti-Ro antibodies, antibodies typical for SLE. This finding suggests that molecular mimicry plays an important role in the development of SLE.[16,17,18]

The type of infection also influences the clinical manifestation. Exposure to EBV is associated with a mild form of SLE, manifested in skin and joint involvement.[19] In contrast, an infection with rubella is more likely to lead to neuropsychiatric manifestations.[20]

Furthermore, the influence of drugs has been discussed; administration of certain drugs can lead to the development of the so-called “Drug Incuded Lupus Erythematosus” (DILE). Among these drugs are antiarrhytmics, anticonvulsants, diuretics, statins and medication for

hypertension, psychosis and infections.[21] In such cases, patients develop a relatively mild form of lupus with arthritis and skin involvement. Termination of drug usage eliminates the disease. Biologicals such as anti-TNFα, which, are a new approach to treat SLE, paradoxically can cause a more troublesome form of DILE, including kidney and central nervous system involvement.[21,22]

Recent studies discussed more environmental triggers such as silicone implants, pesticides, cleaning solvents, hair dyes and phytoestrogens.[21]

1.1.2.4 The influence of sex hormones

Looking at the epidemiologic data, we clearly see that SLE is a disease, which mostly young females suffer from.

Research shows that estrogen receptors can be found on a variety of immune cells such as B- and T-lymphocytes or macrophages.[23] Consequently, estrogens are able to modulate the function of these cells. One such mechanism is the modulation of the expression of CD22, a surface protein on B-cells that inhibits B-cell receptor signaling. The influence of estrogens on CD22 expression can result in the escape from negative selection of auto-reactive B cells.[24] In the T-cell lineage, estrogens stimulate the Th2 response and support the survival of auto-reactive T-cells.

Furthermore, prolactine and progesterone influence the immune response by also leading to the survival of auto-reactive lymphocytes.[23] Testosterone, however, seems to have protective, anti-inflammatory effects; studies showed reduced plasma levels of testosterone in female SLE patients.[25]

Patients suffering from Klinefelter syndrome (47, XXY) have a higher risk to develop SLE, whereas women with only one X-chromosome (Turner syndrome) seldomly develop SLE.[26,23]

1.1.2.5 Immunopathology

All of these genetic, epigenetic, environmental and hormonal factors lead to an immunological dysregulation, which may result in the development of SLE. The following deficiencies and abnormalities, partially mentioned above, are central in disease development:

1) Abnormal cell function and cytokine levels
2) Autoantibody production and immune complex deposition
3) Complement deficiencies and impairment of phagocytosis

1) Abnormal cell function and cytokine levels

Central to abnormal cell function seen in SLE are the presence of hyper-reactive B-lymphocytes and an imbalance in the effector and suppressor T-cell ratio. In addition, serum levels of cytokines, crucial proteins for cell signaling, are deviant.

Serum levels of Interleukin 6 (IL-6), a pro-inflammatory cytokine, are elevated in SLE patients compared to healthy controls. IL-6 enhances B-lymphocyte maturation into plasma cells and enhances autoantibody production. IL-6 further inhibits the suppressive function of regulatory T-cells and promotes the resistance of effector T-cells to regulatory T-cells (Treg), thus leading to an inflammatory environment.[27,28]

Another effect that IL-6 exerts on Treg is the conversion of Treg into IL-17 producing cells (in the presence of TGFß). IL-17 facilitates effector T-cell activation and tissue infiltration by upregulating intracellular adhesion molecules (ICAMs).[29] An imbalance in the Th17/Treg ratio favoring Th17 lymphocytes is seen in SLE patients.[30] Interestingly, elevated IL-6 levels correlate positively with disease activity.[31] High urinary IL-6 excretion was seen in patients with active proliferative lupus nephritis and elevated IL-6 levels were found in the cerebrospinal fluid in patients with an ongoing neuropsychiatric manifestation.[28,32]

Impaired suppressive capacity of Treg is also reported with high levels of TNF-alpha and Interferone-alpha[33,34]

Similar to IL-6, IL-10 has pro-inflammatory effects, as it functions as stimulator for B-cell proliferation and differentiation, and is elevated in patients suffering from SLE. However, since it also shows anti-inflammatory effects through the suppression of effector T-cells and promotion of Treg differentiation, it remains unclear wheter IL-10 is has beneficial or detrimental effects on SLE.[27,29]

Transforming growth factor ß TGFß and IL-2 are the most relevant cytokines for Treg differentiation. In SLE, serum levels of both cytokines are reported to be reduced, hence leading to the dominance of effector T-cells and the potential of an overreacting immune system.[35]

2) Autoantibody production and immune complex deposition

As previously mentioned, patients suffering from SLE show hyper-reactive B-lymphocytes, leading to the production of autoantibodies.

Directed at self-molecules in the nucleus, cytoplasm or on the cell surface, these autoantibodies either act directly pathogenically or form immune complexes with soluble

molecules that deposit in various organ sites. Consequently, these autoantibodies lead to tissue damage through activation of inflammatory pathways. Interestingly, the occurrence of specific autoantibodies is associated with different organ manifestations.

Although a multitude of autoantibodies has been described in SLE, only a few of these are helpful for diagnosis and prognosis.

Antinuclear antibodies (ANA) target nuclear and cytoplasmic antigens. ANA are a key indicator in SLE, but they are also known to be found among healthy individuals, and further, their levels can be increased among elderly sick individuals.[36,37] ANA can be divided into subgroups characterized by the specific antigen they target. Hereby, antibodies against double-stranded DNA (dsDNA), Ro/La, Sm and Ribosomal P need to be mentioned.

The anti-dsDNA antibody is one of the most specific autoantibodies in SLE, yet shows low sensitivity since it is only present in 50-60% of lupus patients and can occur transiently. This antibody has a high prognostic value, as increasing serum levels correlate with disease activity and exacerbation of lupus nephritis.[38,39]

Another highly specific autoantibody (>98% specifity), which is found in 5-30% of SLE patients, is the anti-Sm autoantibody. Like anti-dsDNA, anti-Sm is associated with lupus nephritis, but has no correlation with its severity.[39,40] The presence of either of these two antibodies is established as a criterion under the current ACR criteria for classification of SLE.

Anti-Ro and anti-La autoantibodies are not specific antibodies for SLE and are primarily associated with Sjögren´s syndrome (prevalence of 90%). They do, however, remain useful for SLE diagnosis when anti-dsDNA or anti-Sm are absent.[39]

Antibodies targeting cytoplasmic ribosomal P also show high specifity, but can only be detected in a small group of SLE patients (12-16%). Studies found an association with renal and hepatic involvement; a possible association with neurological involvement is still being debated.[39,41]

3) Complement deficiencies and impairment of phagocytosis

The complement system is attributed with an important role in the clearance of immune complexes and apoptotic debris, and in the recognition and elimination of pathogens. A failure to remove any of these components can lead to an accumulation of waste material within tissues and thus, potentially evoke an autoimmune response.[42]

A hereditary homozygous deficiency in the classical pathway of the complement system is linked to an increased susceptibility of SLE. Over 90% of individuals with a deficiency in

C1q and C1r/C1s develop SLE or a SLE-like disorder; additionally 75% of those with C4 deficiency develop SLE. Depending on the missing complement factor the severity of SLE can be estimated: Deficiencies of C1q and C4 lead to a rather severe form of SLE, whereas a deficiency of C2 is associated with milder symptoms.[42,43,44]

1.1.3 Clinical manifestation and diagnosis

Aside from Sjögren´s syndrome, SLE is the most common among connective tissue diseases. Yet it remains a rare disease. Prevalence rates range from 20-70 per 100,000 and incidence rates range from 1-10 per 100,000 per year. SLE predominantly affects women with a female to male ratio of 9:1.[45]

The disease exhibits a broad range of symptoms and manifests itself differently among patients. In most cases, the disease progresses along a typical cyclical course, characterized by manifestation at different organ sites and by a variable level of disease severity. Some patients, however, suffer from a chronic progressive course. The diagnosis of SLE often proves difficult. Unspecific symptoms such as fever, weakness or weight loss can be the first signs of the disease. Upon the appearance of typical skin lesions (e.g. “butterfly rash”), arthralgia, or involvement of inner organs, the diagnosis “SLE” must be taken into consideration.

The following list summarizes the broad range of symptoms of the disease:

- Nonspecific symptoms such as fever, weakness, weight loss and lymphadenopathy
- Musculoskeletal manifestations: arthralgia, arthritis, myalgia, myositis
- Dermatological manifestations: malar rash (“butterfly rash”), photosensitivity rash, oronasal ulcerations, alopecia, secondary Raynaud-syndrome, vasculitis
- Cardiopulmonary manifestations: pleuritis, pericarditis, endocarditis, myocarditis, pneumonitis, pulmonary infiltration
- Neuropsychiatric manifestations: depression, epilepsy, psychosis, anxiety disorders, multiple sclerosis-like symptoms
- Renal manifestations: proteinuria, hematuria, nephrotic syndrome, elevated serum creatinine
- Gastrointestinal manifestations: dysmotility, abdominal pain, nausea, vomiting
- Hematologic manifestations: anaemia, leucopenia, thrombocytopenia

A positive diagnosis is based on the recognition of clinical symptoms and laboratory tests. For diagnostic assistance, the criteria for classification of SLE by the American College of Rheumatology (ACR) are commonly used. It must be considered that these criteria are intended for classification in research studies and do not establish an absolute standard for the diagnosing of individual patients. If at least 4 of the 11 symptoms are present, the diagnosis 'SLE' is very likely.

In 2012 Petri *et al.* undertook a revision leading to the SLICC criteria (Systemic Lupus International Collaborating Clinic criteria). These criteria showed higher sensitivity (97% vs. 83%), but lower specifity (84% vs. 96%) compared to the current ACR criteria.[46] The SLICC-classification also requires at least 4 criteria (with at least 1 clinical and 1 laboratory criterion) or biopsy-proven lupus neprhtis with ANA or anti-dsDNA antibodies.

Table 1.1. Overview of the ACR criteria and SLICC criteria

	#	ACR-criteria description	#	SLICC-criteria description
Ulceration	1	Oral or nasopharyngeal ulceration	1	Oral or nasal ulceration
CNS manifestation	2	Seizures or psychosis	2	Seizures, psychosis, mononeuritis multiplex, neuropathy, myelitis, acute confusional state
Hematologic manifestation	3	Hemolytic anemia, or Leucopenia, or Lymphopenia, or Thrombocytopenia	3	Hemolytic anemia
			4	Leukopenia, or Lymphopenia
			5	Thrombocytopenia
Renal manifestation	4	Persistent proteinuria, or cellular casts	6	Persistent proteinuria, or cellular casts
Cardial/pulmonal manifestation	5	Pleuritis, or pericarditis	7	Serositis
Arthritis	6	Non-erosive arthritis	8	Synovitis, or tenderness and morning stiffness
Photosensitivity	7	Photosensitivity		
Alopecia			9	Non-scarring alopecia
Skin manifestation	8	Malar rash	10	Acute cutaneous lupus
	9	Discoid rash	11	Chronic cutaneous lupus
Immunologic disorder	10	Anti-DNA, or anti-Sm, or positive finding of antiphospholipid antibodies	*	1) ANA 2) Anti-dsDNA 3) Anti-Sm 4) Antiphospholipid antibody 5) Low complement 6) Direct Coombs test
	11	ANA		

* Laboratory criteria of the SLICC criteria

In the course of making the diagnosis it must be considered that symptoms, especially skin manifestations, indicating SLE can also occur in other subforms of lupus erythematosus or connective tissue diseases. These include chronic cutaneous lupus erythematosus (CCLE), subacute cutaneous lupus erythematosus (SCLE), drug-induced lupus erythematosus (DILE), dermatomyositis and mixed connective tissue disease (MCTD).[47,48]

1.1.3.1 Lupus arthritis

Arthritis is often reported as a first symptom of SLE and is seen in up to 95% of patients over the course of the disease.[49] Even though joint involvement is a non-life-threatening condition, patient surveys reveal arthritis as a major burden of SLE patients leading to impairment and daily life hurdles.[50,51]

The nature of SLE arthritis is variable ranging from mild arthalgia to deforming arthritis. In most cases it presents as a symmetric non-erosive and non-deforming polyarthritis of the small joints, especially of the hand including the metacarpal phalangeal (MCP), proximal interphalangeal (PIP) and distal interphalangeal (DIP) joints. Lupus arthritis predominantly is non-erosive in projection radiography; nevertheless, in a small population of patients, erosive, deforming arthritis is seen, that resembles rheumatoid arthritis (often referred as “rhupus”).[48] Interestingly, and MRI-based study reveals bony erosions in 93% of examined wrists and 64% of examined MCP joints from patients suffering from lupus arthritis; this study gives rise to the notion that minimal erosive arthritis in SLE may be much more prevalent than assumed.[52]

1.1.4 Disease assessment

Monitoring and assessing the course of SLE is necessary for the successful management of patients suffering from the disease. A suggested framework for assessment consists of four essential components: (1) accurate diagnosis, (2) monitoring of disease activity, (3) recording of accumulated damage and (4) integration of these with the patient’s own perceptions of health status and quality of life.[53] For each of these components multiple scores have been developed. The importance of such framework lies in its ability to not only identify the progression of SLE at the level of the individual and thus improve treatment approaches, but it also helps to develop possible standards of comparison between patients.

For assessing disease activity, many measures have been developed and are constantly validated. These include indices, such as:

- Systemic Lupus Erythematosus Disease Activity Index [SLEDAI],
- British Isles Lupus Assessment Group Index [BILAG]),
- Euopean Consensus Lupus Activity Measure [ECLAM],
- Systemic Lupus Activity Measure [SLAM], and
- SLE Activity Index Score [SIS]).[54]

Predominantly clinical features, but also laboratory parameters are used in these scores. All mentioned activity scores perform with high reliability and validity. However, there are some differences that may make one index a more useful tool in a particular situation.[48]

Accumulating damage from the disease itself, the treatment and from co-morbidities can be assessed with the SLICC/ACR Damage Index (SDI). A higher SDI in an early stage of the disease has been associated with a poor prognosis and with increased mortality.[53] In 2010, Costenbader *et al.* published an alternative score, called the Lupus Damage Index Questionnaire (LDIQ).[55] Unlike the SDI, this score does not need the direct assessment from a physician since it can be self-assessed by the patient. This test, however, still needs to be validated for use in longitudinal studies, and its reliability among patients needs to be evaluated.

To assess the patient's own perception of his/her health and quality of life, Short-Form-36 (SF-36) is often used. This survey helps establish limitations in everyday life, pain, vitality and general health perceptions of the patient. SF-36 is a common survey applicable in various chronic diseases, and can be combined with more detailed SLE-specific questionnaires (e.g. Systemic Lupus Erythematosus Quality of Life [SLEQoL]).[53]

1.1.5 Treatment

The treatment possibilities of SLE consist of a very limited number of drugs: Only glucocorticoids, hydroxychloroquine, immune suppressants such as azathioprine or cyclophosphamide, and very recently belimumab have been approved as therapy options.

The guidelines for SLE treatment were updated in 2008 by the European League Against Rheumatism (EULAR), and in 2012 more recent guidelines regarding treatment of lupus nephritis were published by the American College of Rheumatology.[56,57]

The therapy of choice is dependent on the level of disease activity. Mild cases of SLE (showing no major organ manifestation) are primarily treated with glucocorticoids and antimalarials, paired with limited, short-term doses of nonsteroidal antiinflammatory drugs (NSAID). If this treatment is not satisfactory, the immune suppressants mycophenolate mofetil (MMF) or azathioprine should be considered. In cases with involvement of major organs, therapy should begin with cyclophasphamid or MMF, paired with glucocorticoids (induction phase), and later switching to azathioprine or MMF (maintenance phase). For refractory disease, additional therapy strategies such as intravenous immunoglobulin (IVIG) or plasmapheresis are suggested.[56,57,58]

Biologicals hold much promise in SLE therapy. Belimumab, a recombinant monoclonal antibody against BLys, was the first and still remains the only biological being approved for SLE treatment. Belimumab was approved in 2011 for cases of refractory disease, and should be used in combination with the above mentioned treatment options.[59,60] Following the success of Belimumab hopes are high that similar therapies may be approved for therapy.

The treatment and prevention of co-morbidities form another considerable point in SLE therapy. To prevent osteoporosis in patients receiving long-term glucocorticoid therapy the EULAR recommends vitamin D and calcium for women who may become pregnant, and bisphosphonate for postmenopausal patients. Since SLE patients have higher risks of developing hypertension, atherosclerosis, dyslipidaemia, diabetes and malignancies (especially non-Hodgkin's lymphoma), these co-morbidities have to be specifically monitored and, if found, must be treated. Sun-protection should also be considered to prevent skin lesions and systemic flares. Furthermore, lifestyle modifications such as weight control, physical exercise and smoking cessation are recommended by the EULAR.[56,58]

1.2 Regulatory T-cells

1.2.1 General aspects and brief history

The immune system not only detects and defends against pathogens such as bacteria and viruses, but it also constructs strategies to control responsiveness to self-antigens, and thereby avert the risk of autoimmunity. One mechanism for the latter function is the suppression of auto-reactive T-lymphocytes. In 1970 Gershon and Kondo first suggested that T-cells themselves have the capability of suppressing the proliferation of other T-cells.[61] Upon this finding many studies arose addressing this topic, but soon interest in the so-called suppressor T-cells diminished due to reasons such as a failure to detect reliable markers for distinguishing suppressor T-cells from other T-cells.[62] However, in the 1990´s Sakaguchi *et al.* found that neonatal thymectomy (NTx) in mice led to the elimination of $CD25^+$ T-cells, and further, that the inoculation of $CD25^+$ T cells shortly after NTx prevents autoimmunity.[63] Thus a first marker to identify T-cells with suppressive capabilities was established, and subsequently these cells were called regulatory T-cells (Treg).

1.2.2 Subpopulations and markers for identification

Treg form a heterogeneous group of T-lymphocytes; the majority of Treg develop from $CD4^+$ T-cells. $CD4^+$ Treg can be classified as "natural Treg" (nTreg) or "induced Treg" (iTreg). nTreg obtain their phenotypical differentiation in the thymus and emigrate to the periphery as $CD4^+CD25^+$ cells. iTreg, in contrast, are $CD4^+$ single positive cells which emigrate from the thymus into the peripheral lymphoid tissues, where they differentiate upon exposure to antigen.[64] iTreg make up a range of cells including transforming growth factor ß (TGFß)-expressing T-helper 3 (Th3) cells and IL-10 producing T-regulatory 1 (Tr1) cells. The peripheral $CD4^+CD25^+$ Treg population, which accounts for 1-2% of the peripheral $CD4^+$ population, is a combination of natural and induced Treg.[65]

Helios, a recently found transcriptional marker, is only expressed on nTreg. Not only is it helpful in distinguishing nTreg from iTreg, but more importantly, it also takes part in regulating nTreg expression by modulating Foxp3 promotor genes.[66]

Although Sakaguchi *et al.* defined CD25 as a marker only occurring on Treg, more recent studies have proven that CD25 is also expressed on activated non-regulatory $CD4^+$ T-cells.[67] $CD4^+$ T-cells showing high expression of CD25 are associated with *in vitro* suppressing

activity and therefore are characterized as Treg ($CD4^+CD25^{high}$ cells), whereas cells with low expression of CD25 are considered activated $C4^+CD25^{low}$ T-cells.

Crucial for the development, maintenance and function of Treg, the major transcription factor "forkhead box protein 3" (Foxp3) proves to be another essential marker for phenotypical characterization. So-called *scurfy* mice, which lack Treg due to a genetic defect in Foxp3, develop a fatal multi-organ autoimmune disease with many resemblances to human SLE.[114] In the human body, mutations in genes encoding for Foxp3 cause the immunodeficiency syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome), which is manifested in various autoimmune disorders such as type I diabetes, thyroiditis, inflammatory bowel disease, and allergies.

Foxp3 is currently regarded as the most specific intracellular marker for Treg in mice.[68] In humans, however, studies found that $CD4^+CD25^{low}$ T-cells are also capable of expressing Foxp3, and further, that T-cell activation can induce transient expression of Foxp3.[69,67] Thus, in humans, Foxp3 does not exclusively identify Treg. Bonelli *et al.* demonstrated that increased SLE disease activity is associated with a decrease in $CD4^+CD25^{high}$ T-cells and an increase in $CD4^+Foxp3^+$ T-cells.[70] A substantial portion of the elevated $CD4^+Foxp3^+$ cells did not express CD25 ($CD4^+CD25^-Foxp3^+$). These cells phenotypically resemble $CD4^+CD25^+Foxp3^+$ Treg rather than activated T-cells. *In vitro* functional analysis showed that these cells are similar to Treg as they exert a considerable suppression of T-cell proliferation.[71]

Various other markers for Treg classification have been described, including the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), glucocorticoid-induced TNF-receptor (GITR), lymphocyte activated gene-3 (LAG-3), neutropilin-1 (Nrp1), $CD127^{low}$, $CD62L^{high}$, CD39 and CD72.6> These markers, however, are not exclusive to the Treg phenotype and the functional significance of many of these markers remains unclear.

Apart from the $CD4^+$ Treg population, other suppressor T-cell populations have been identified, such as $CD8^+$ regulatory T-cells and natural killer like T-cells (NKT).[72]

Difficulties in isolating these cell types and a lack of specific markers for characterization, results in limited data concerning cell properties and mechanisms of suppression.

1.2.3 Functions of Treg

Treg have the capability to suppress the activation, proliferation and function of a large number of immune cells, including $CD4^+$ and $CD8^+$ effector T-cells, natural killer cells (NK), B-cells, as well as antigen-presenting cells (APC). In the following, Treg effects on T-cells and antigen-presenting cells will be discussed.

1.2.3.1 Effector T-cell targeting mechanisms

Multiple mechanisms with which Treg suppress effector T-cells have been described. One mechanism is the modulation of the immune system via cytokines, including IL-10 and TGFß. Several *in vivo* studies report that IL-10 and TGFß production by Treg is essential for the prevention of autoimmune disorders, such as inflammatory bowel disease.[73,74] Although IL-10 and TGFß are generally seen as major suppressive cytokines, their detailed function in the Treg-linked suppression is still debated as findings from *in vitro* studies deviate from those of *in vivo* studies. Unlike the mentioned *in vivo* models, data from *in vitro* experiments do not confirm an essential role of IL-10 or TGFß in Treg dependent suppression mechanisms. Neutralization of either of these cytokines does not inhibit *in vitro* suppression.[75]

Cytolysis of target T-cells is another potent mechanism of Treg-mediated suppression. *In vivo* studies reported that human Treg are able to express granzyme B, a serine protease which leads to a rapid induction of apoptosis.[76] *In vitro* experiments showed, that upon stimulation with CD3 and CD46, Treg express granzyme A and thereby kill activated $CD4^+$ and $CD8^+$ T-lymphocytes.[77]

Another potential mechanism for Treg-mediated suppression is the deprivation of essential cytokines. IL-2 is vital for growth, proliferation, and differentiation of effector T-cells. Moreover, IL-2 is also required for Treg survival and *de novo* induction from naïve T-cells. Treg thus compete with effector T-cells for IL-2 and thereby disrupt the activation of effector T-cells.[78]

1.2.3.2 APC targeting mechanisms

As already mentioned, apart from targeting T-cells, Treg can also modulate APC such as dentritic cells (DC). The main function of APC is the processing and presenting of antigen material to naïve T-cells, which then leads to the activation and differentiation of $CD4^+$ and

$CD8^+$ effector T-cells. Consequently the down-regulation of APC activity leads to a reduced activation rate of effector T-cells.

The interaction between cytotoxic T-lymphocyte antigen 4 (CTLA-4; found on Treg) and CD80 and CD86 (found on dendritic cells) is an important pathway by which Treg mediate their suppressive function. Modulation of CD80 and CD86 by Treg may stimulate the expression of the enzyme indoleamine 2,3-diosygenase (IDO), which produces compounds toxic to T-cells neighbouring DCs.[75] Defects in this pathway can cause severe damage; a study by Wing *et al.*, showed that a loss of CTLA-4 expression led to the development of a fatal T-cell-mediated autoimmune disease in mice.[79]

Another antigen that has recently been identified as being involved in Treg cell suppression is LAG-3. LAG-3, a cell surface molecule on Treg binds to the MHC class II on DCs and thus, via an inhibitory signaling pathway, can down-regulate the maturation of these cells.[80]

1.2.4 Treg and SLE

1.2.4.1 Treg numbers and functions in SLE patients

Controversial data has been published concerning Treg numbers in the peripheral blood of SLE patients. Most studies report reduced Treg levels in SLE patients compared to healthy controls (HC).[68,81,82] Nevertheless, also undisturbed as well as increased Treg numbers have been observed.[83,84] Such deviation in observation may be attributed to testing during different stages of disease activity, differentiation in disease manifestation, or different isolation techniques (i.e. FACS sorting vs. Treg isolation using magnetic beads). Furthermore, ongoing therapy may also contribute to differences in observation; treatments using glucocorticoids, Rituximab or plasmapheresis, result in an increased number of $CD4^+CD25^{high}$ T-cells.[64]

In addition, since a specific marker for human Treg has yet to be identified, using different markers for Treg phenotyping may also have led to these controversial results.[85]

Treg dispersion throughout the body has also been considered an influencing variable on the number of Treg observed in the blood. However, a study by Miyara *et al.* showed that the reduced amount of circulating Treg is not due to accumulation in lymphoid tissue or SLE-involved organs.[86]

Apart from quantitative analyses of Treg in SLE patients, studies focused on the qualitative function of these cells. These studies also present diverse results. Some researchers observed

changes in the suppressive function of Treg, whereas other studies suggest an increased resistance of effector T-cells to Treg-mediated suppression. Other studies fail to confirm either of these findings.[87,88,89] In regards to experiments from our laboratory, Bonelli *et at* showed that naturally occurring $CD4^+CD25^+FoxP3^+$ Treg are reduced in both number and function in patients with active SLE.[68]

1.2.4.2 The therapeutic potential of Treg

As a consequence of ever increasing knowledge concerning Treg and their pivotal role in immune homeostasis, great interest has arisen in the therapeutic potential of these cells. Several studies in mice have proven Treg to be capable of preventing the development and progression of autoimmune diseases, including autoimmune gastritis, type 1 diabetes and inflammatory bowel disease.[90,91] Regarding SLE, Scalapino *et al.* reported that adoptive transfer of cultured Treg into lupus-prone mice reduced the development rate of renal disease. Furthermore, an additional transfer of Treg after treated mice had developed proteinuria slowed the disease progression and significantly extended the mean life expectancy.[92]

In a previously conducted pilot experiment from our research group, we could show that regulatory T-cells that were extracted from spleens (nTreg) can ameliorate both the clinical and histological course of pristane-induced lupus.[93]

The first of their kind, recent clinical trials tested the therapeutic potential of Treg in a small sample size of patients. These trials reported promising results: Adoptive transfer of *ex vivo*-expanded Treg sucessfully treated graft-versus host disease (GVHD).[94,95] Another recently published study demonstrated that Treg are capable of delaying the onset of Type I diabetes in children.[96]

Different strategies for Treg-therapy have been designed. Adoptive transfer of *ex-vivo* expanded Treg forms an attractive approach. In this strategy, Treg are isolated from the patient, enriched, expanded, and reinfused. This approach allows for exact phenotypical analysis prior to infusion and control over the dosage of cells infused.[97] Another strategy is the administration of agents which augment Treg activity. Regrettably, a clinical trial that used anti-CD28 antibodies to stimulate Treg differentiation, led to a massive cytokine storm and multi-organ-dysfunction in healthy adults.[98] This approach demonstrated, that the clinical implementation of Treg-therapy remains challenging and that many barriers still have to be overcome to use Treg in the daily routine.

1.3 Pristane

1.3.1 General aspects

The hydrocarbon 2,6,10,14-tetramethylpentadecane, commonly known as pristane, is a ubiquitous natural compound derived from chlorophyll. Found in many plants, pristane is especially concentrated in marine organisms, including algae, zooplankton and higher animals such as sharks or other fish. Pristane is also found in coal and ancient sediments, and is a common constituent of mineral oils.[99]

Pristane and mineral oils are known to induce autoimmune disorders. In New Mexico, USA, a study reported significantly higher instances of SLE and rheumatic diseases in a community living upon the site of a former oil field. In blood samples from exposed individuals, significantly higher levels of pristane were detected. Interestingly, each subject, in whom detectable levels of mineral oils were found, suffered from lupus or symptoms associated with disorders of the immune system.[100]

We encounter hydrocarbons on a daily basis. Mineral oils are used in cosmetics, laxatives and as a protective coating on food. The dietary uptake of mineral oil is estimated at 9-45 grams per year. This uptake is believed to be responsible for the development of lipogranulomas, which are found in several organs like the liver, spleen, and lymph nodes of healthy individuals in developed countries.[101] The clinical significance of these formations is not known, but its generally believed that their presence is not associated with an inflammatory response.[102] In contrast, accidental aspiration of mineral oil (e.g. aerosolized industrial material) can cause a severe chronic pneumonitis, termed “lipoid pneumonia”, which shows inflammatory lesions of the lung. These lesions closely resemble murine lipogranulomas, which occur on peritoneal surfaces after intraperitoneal injection of pristane. Histological analysis of these lipogranulomas showed, that they are formed due to a chronic granulomatous inflammation around the deposit of the mineral oil. Macrophages engulf the mineral droplets and draw inflammatory cells like neutrophils and lymphocytes to the inflammatory site.[102,103]

1.3.2 Pristane induced lupus and other mouse models for SLE

Upon intraperitoneal injection of pristane, mice develop an autoimmune response that closely resembles SLE. This so-called pristane induced lupus (PIL) is characterized by autoantibody

production and SLE-like organ-manifestation such as arthritis, pneumonitis and glomerulonephritis.

Autoantibody production changes over the course of time, and shows interstrain variability in their frequency and levels. Regarding BALB/c mice, anti-histone and anti-chromatine antibodies appear within the first month after injection, followed by the development of anti-Sm antibodies after 3 months. The majority of mice treated with pristane developed antibodies (100% anti-chromatin, 93% anti-histone, 80% anti-Sm) after 8 months.[104]

Concerning kidney involvement, BALB/c mice develop severe proteinuria and nephritis upon pristane injection. Histological analysis of kidneys from affected mice showed segmental or diffuse proliferative glomerulonephritis, paired with glomerular deposition of immune complexes.[105] C57BL/6 mice, in contrast, develop a milder course of kidney involvement.[101]

Upon pristane injection, arthritis develops only in a limited number of mouse models. C57BL/6 and NZB/W F1 mice did not show clinical or histological signs of arthritis. BALB/c mice are susceptible to developing PIL-arthritis. First clinical signs of arthritis develop 3 to 4 months after pristane injection. Histological analysis at 8 and 12 months show manifestation rates of 58% and 75%, respectively.[104]

PIL arthritis in BALB/c has proven to be a useful mouse model to study human lupus arthritis; a previously conducted study from our research group demonstrated that about 60% of PIL-mice develop erosive arthritis, an additional 16% presented with clinical signs without a histological correlate, while 27% did not develop either form. Thus, the joint involvement in PIL does resemble human SLE, since there are non-affected, transiently affected, as well as highly affected animals.[93, 104] Compared to the histological picture of rheumatoid arthritis, erosions in PIL arthritis are more superficial and rarely affect both cortical layers.[93]

Another manifestation of PIL is hemorrhagic pulmonary capillaritis, characterized by perivascular infiltration of immune cells and endothelial injury. While lung involvement is seen in only some SLE patients, it affects almost every PIL-mouse. Therefore, PIL seems to be a good disease model to also assess SLE lung involvement.[106]

In summary, PIL is a useful mouse model for studying human SLE as it meets several criteria of the ACR classifications for SLE diagnosis. Although PIL shows many advantages (e.g. presents a broad spectrum of lupus-like autoantibodies; cost-effective), disadvantages, such as a rather long period of disease development, and the limited penetrance of some organ manifestations have to be considered.

Several other SLE mouse models exist, each showing varying subsets of symptoms that resemble human SLE. These mouse models can be divided into two main categories, namely, spontaneous (disease development upon genetic susceptibility) and induced models. The most used models of spontaneous lupus include the F1 cross between New Zealand Black (NZB) and New Zealand White (NZW) strains (NZB/NZW F1), and its derivatives, the MRL/lpr, and the BXSB/Yaa strains. Induced mouse models include the already mentioned PIL, and the chronic graft-versus-host-disease model (cGVHD). Both of these categories provide important insight into the pathogenesis of SLE; while spontaneous models allow for the identification of susceptibility loci and thus aid in establishing the genetic background of SLE, induced models are crucial in identifying the cellular mechanisms involved in SLE.[107,108]

1.4 BALB/c mice

The BALB/c strain is a widely used mouse model in animal experimentation. The origin of this strain goes back to the early Nineteen hundreds; In 1913 Halsey Bagg developed the "Bagg albino" from a stock provided by an Ohio pet dealer. Throughout the following decades, several researches worked to refine the stock and subsequently, by 1932, Snell had created the BALB/c strain. Over the next decade several laboratories developed subsets of the BALB/c strain (e.g. BALB/cAnN, BALB/cJ, BALB/cWt). Resulting from spontaneous mutations, these subsets differ significantly in their disease susceptibility and behavior. For example, sub-strains derived from the Andervont lineage typically develop plasmacytomas, while BALB/cJ, a strain from the Scott subline, are resistant to plasmacytoma development. In addition, the BALB/cJ strain exhibits more aggressive behavior than the BALB/cAnN of the Andervont family.[109]

The BALB/c strain is used in many disciplines, but especially in immunological and cancer research. BALB/c mice are particularly sensitive to carcinogens, and spontaneously develop cancer later in life, including renal tumors, reticular neoplasms, and primary lung tumors. Usually BALB/c mice are not prone to develop SLE. Intraperitoneal injection of pristane, however, leads to the development of plasmacytomas and PIL, thus BALB/c often serve as a strain for the study of human SLE.[110]

2 Objective for the current investigation

The ability of Treg to suppress the activity of immune cells and thereby ameliorate tissue damage has placed these cells into the spotlight of research.
The transfer of Treg has not only been effectively tested in multiple animal studies, but also has already been successfully conducted in patients.[97] Nonetheless, the clinical implementation of Treg remains challenging and further investigations need to be done to prove these cells to be an effective and safe therapeutic agent. Regarding SLE, clinical trials addressing the adoptive transfer of Treg within humans have not been performed yet. Animal testing continues to be crucial in evaluating Treg therapy in SLE.

In our study we investigated the therapeutic potential of *in vitro* induced Treg in mice suffering from PIL. The primary end point was to assess, if Treg are capable of reducing PIL-arthritis. Comparing the clinical course and histology of Treg treated PIL-mice to PIL-mice without Treg treatment, we estimated the influence of Treg on the disease severity.

We therefore evaluated the following questions:

- Does a single intravenous boost of $5x10^6$ Treg at the time of disease induction reduce PIL arthritis?
- Do monthly intravenous injections of 10^6 Treg reduce PIL arthritis?
- To what extend are Treg capable of reducing PIL arthritis? Do they retard the onset? Do they ameliorate the clinical course and activity of the disease?
- Does the clinical course correlate with the histology of paws?

3 Materials and Methods

The study consisted of two independent experiments, which were approved by the ethics committee of the Medical University of Vienna and is in accordance with the Protection of Animals Act 2012 (BGBI. I. Nr.114/2012).

3.1 Mice and disease induction

For the study female 6-8 week old BALB/cAnNCrl mice were obtained from Charles River Laboratories.

Mice received an intraperitoneal injection of 0.5ml pristane for disease induction (PIL-group) or 0.5 ml of phosphate buffered saline (PBS), the latter being used as healthy-controls (HC-group). On the following day, a fraction of mice receiving pristane, were injected intravenously with either (a) 10^6 Treg and received additional equivalent doses every four weeks thereafter or (b) with a single boost of $5x10^6$ Treg. Thus, 4 groups were obtained within the two experiments:

Table 3.1. Mice groups from the two individual experiments

Group name	Injected substance	Specimen totals
PIL	0.5ml pristane once	21
Treg-rep	0.5ml pristane once, 10^6 Treg monthly	6
Treg -boost	0.5ml pristane once, $5x10^6$ Treg once	8
Healthy Controls (HC)	0.5ml PBS once	6

3.2 Induction and determination of $CD4^+CD25^+Foxp3^+$ iTreg

3.2.1 Isolation of naïve thymocytes

Two 6-8 week old BALB/c mice, also obtained from Charles River Laboratories, were used per month to attain naïve thymocytes. Under sterile labor conditions, thymi were extracted and filtered through a 70µl micro filter in a petri dish filled with 5ml PBS. Filters were rinsed with 5 ml PBS and cells were brought into a 50ml Falcon tube. The petri dish was rinsed with 5ml PBS to obtain the remaining cells. Cells were then centrifuged at 400xg for 7 minutes at 4°C. To eliminate erythrocytes, cells were resuspended in 1ml of erylysis for 3 minutes and again centrifuged. For all instances of centrifuging the mentioned settings were used (400xg, 7min, 4°C).

3.2.2 MACS-Separation of $CD4^+CD8^-CD25^-$ thymocytes

To isolate $CD4^+CD8^-CD25^-$ thymocytes from the cell suspension, Magnetic Cell Separation (MACS®) was used. The MACS method allows to separate cell compositions by incubating the cells with magnetic nanoparticles (Beads) that are coated with antibodies against a particular surface antigen. The magnetically labeled cell supension is brought into a column, which is placed in a strong magnetic field. Cells labeled with magnetic Beads are retained within the column, while unlabeled cells will pass through. Depending on the cell-type of interest, either cells which are attached to the column (positive selection) or cells that run through the column (negative selection) can be used.[111]

To conduct MACS separation, cells were resuspended in MACS-Puffer (1ml/thymus), consisting of 5g BSA and 4ml EDTA per 1000ml PBS. Anti-CD25-PE antibodies (20µl/thymus) were added and the cell suspension was put on ice for 10 minutes. Thereafter, the tube containing the cells was filled with MACS-Puffer (~10ml), then centrifuged and resuspended in MACS-Buffer (1ml/thymus). Anti-PE-Microbeads (30µl/thymus) and anti-CD8 Microbeads (100µl/thymus) were added, and the tube was put on ice for additional 15 minutes. Afterwards, cells were centrifuged, resuspended in 300µl MACS-Buffer, and applied to the MACS column that was placed into the magnetic field of the MACS Separator. After the cell suspension had passed the column, the column was washed with 5ml of MACS-Puffer. Through this procedure $CD4^+CD8^-CD25^-$ thymocytes were obtained as a negative selection.

Between work steps, cells were always kept cold using an ice box to avoid onset of apoptosis. The mentioned antibodies and Microbeads were obtained from Milteny Biotec GmbH.

3.2.3 Cell counting and cell culture

To establish the amount of $CD4^+CD8^-CD25^-$ thymocytes per milliliter, 10µl of the cell-suspension was mixed with 10µl of tryptan blue, introduced into a Neubauer hemocytometer and counted under the microscope. Cells then were centrifuged and depending on their quantity, resuspended in cRPMI (~600,000 cells/ml cRPMI). cRPMI consists of RPMI 1640 with 5% heat-inactivated FBS, 2mM L-glutamine, 1mM HEPES, 0.1mM nonessential amino acids, 1mM sodium pyruvate, penicillin/streptomycin, and 2 µl ß-mercaptoethanol. 100U/ml rhIL-2 and 5ng/ml rhTGFß1 were added to the cell suspension. The cells were deposited into a 24-well flat plate, containing plate-bound anti-CD3 (1µg/well) and anti-CD28 (1µl/well). Each well was filled with 1ml of the cell suspension. The plate was stored in an incubator at 37°C overnight.

On the following day, (day 2 of the cell culture), the cells were split and the medium required replenishment. Therefore, the cells were removed from the wells, deposited into a 50ml Falcon tube, and the wells were washed 2 times to obtain the remaining cells. The cells were centrifuged and resuspended in the adequate amount of cRPMI to attain approximately 600.000 cells/well. 100U/ml rhIL-2 was added to the suspension. The cell suspension was deposited into a well void of plate-bound antibodies and was stored in the incubator at 37°C to induce proliferation and differentiation into induced $CD4^+CD25^+Foxp3^+$ Treg.

On day 5, a small amount (~1ml) of the cell suspension was utilized for FACS-analysis. The cell culture was further stored in the incubator.

3.2.4 FACS-analysis

The relative amount of $CD4^+CD25^+Foxp3^+$ Treg was determined by Fluorescence-activated cell sorting (FACS), a specialized type of flow cytometry. With this method, cells can be characterized upon fluorescent characteristics and specific light scattering.[112]

Staining for multiple surface markers and one intracellular marker (FoxP3) was conducted. The cell sample (~1ml) was divided in two 1ml tubes (~500µl/tube), then centrifuged and resuspended in 100µl of PBS containing 1%FBS and 0,1% NaN3. Antibodies for surface staining were added to one tube, with the following concentrations of antibody solution to

PBS: anti-CD25 (1:25), anti-CD4 (1:200), anti-CD69 (1:200) and anti-CD8a (1:50). The other tube was used for negative control, with the following concentrations of antibody solution to PBS: anti-IgG1 (1:25), anti-rt IgG2b (1:50), and anti-rt IgG2a (1:50). In order that the antibodies bind to the cell surface, the suspension was deposited in an ice box for 20 minutes. Thereafter the tubes were filled entirely with PBS, centrifuged and resuspended with Foxp3 Fixation/Permeabilization working solution. The samples then were incubated overnight at 4°C in the dark.

On the following day, the cells were washed and resuspended in 100µl 1x working solution of Permeabilization Buffer. For intracellular staining, an antibody against Foxp3 (1:25) was added to the tube containing the surface staining of Treg. Anti-rt IgG1 (1:200) was added to the other tube. The tubes were stored at 4°C for 60 minutes in the dark, afterwards centrifuged, and filled with 100µl 1x working solution of Permeabilization Buffer to be used for FACS-analysis.

Only populations with over 80% Foxp3$^+$ cells were used for intravenous injection in BALB/c mice. Detailed work steps and preparation of the working solutions are found on the website of eBioscience (www.ebioscience.com).

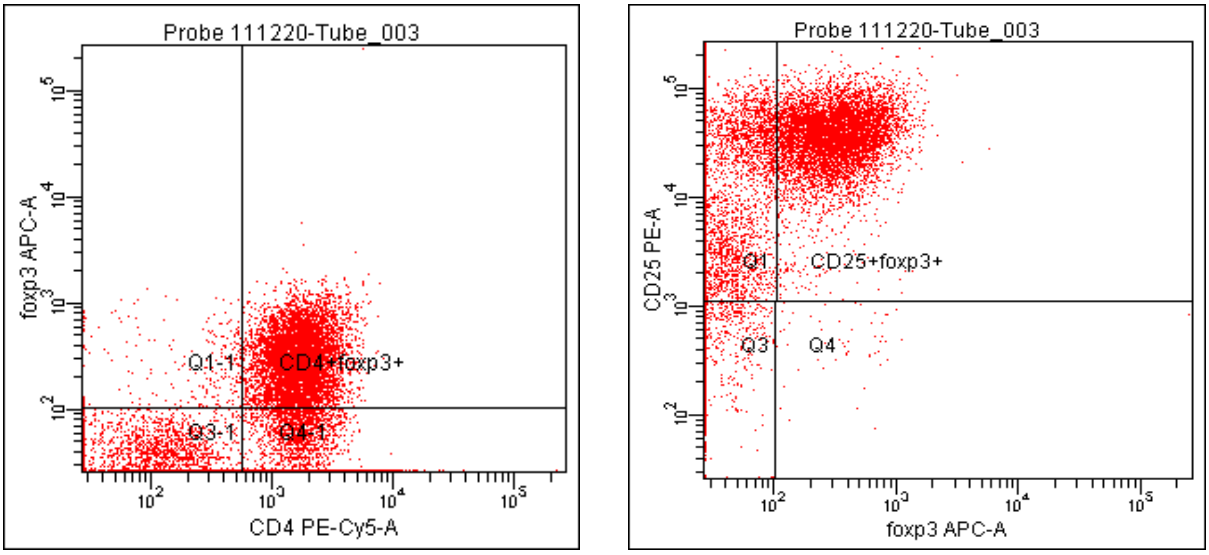

Figure 3.2. FACS-analysis of isolated CD4$^+$CD25$^+$Foxp3$^+$ Treg. Only populations with >80% prurity of CD4$^+$CD25$^+$Foxp3$^+$ cells were used for i.v. injection.

3.3 Intravenous injection of Treg

The cells were harvested on day 6 and resuspended in PBS (10^6 Treg /100µl PBS for each mouse of the Treg rep group, and $5x10^6$ Treg/100µl for each mouse of the Treg-boost group). Mice were anesthetized with an intraperitoneal injection of 100-120µl/mouse of an anesthetic, consisting of Ketasol 100mg/ml, Rompun 20mg/ml and 0,9% NaCl. During sedation, the Treg-cell suspension was introduced through the retro-orbital sinus.

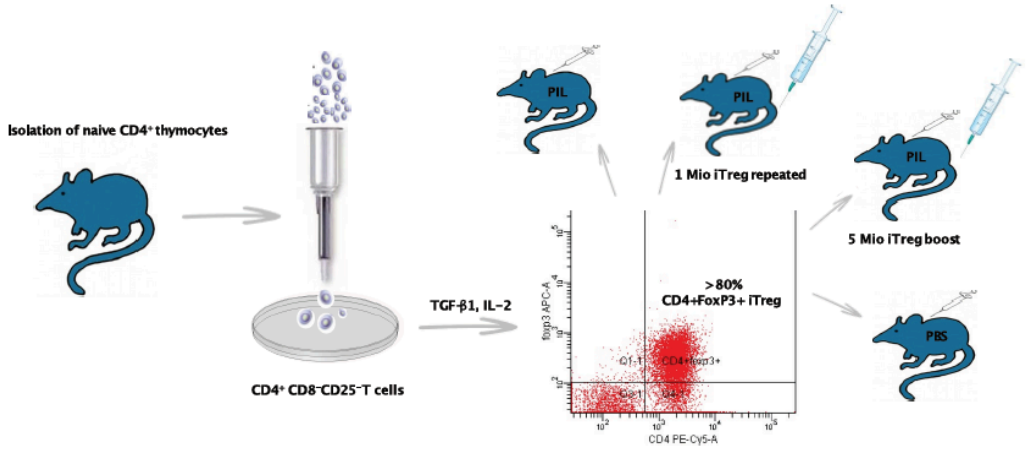

Figure 3.3. Overview of the work steps conducted and mice groups obtained.

3.4 Clinical scoring of arthritis

Every two weeks clinical signs of arthritis were assessed utilizing a well-established semiquantitative score that was originally designed for the assessment of murine models for rheumatoid arthritis.[113] This score consists of the screening for paw swelling and grip strength.

Grade	Paw swelling	Grip strength
0	Normal paw, no swelling	No grip at all
1	Mild swelling of toes and ankle	Severely reduced grip strength
2	Moderate swelling of toes and ankle	Mildly reduced grip strength
3	Severe swelling of toes and ankle	Normal grip strength

Table 3.4. Score to classify paw swelling and grip strength.

3.5 Histomorphometric analysis

8 months following disease induction, mice were euthanized in order to obtain the hind paw of most strongly exhibiting swelling and grip loss. For histological analysis, the paws were fixated up to 8 hours in 10% neutral-buffered formalin and thereafter submerged in EDTA 14% for 5-7 days in order to achieve decalcification. Changing of EDTA was done after 24 and 36 hours. The samples then were embedded in paraffin and cut into 2-µm sections. The obtained slices were stretched in a water bath at 50°C, applied onto a microscope slide, and stained using two solutions: tartrate-resistant acid phosphate (TRAP) stains osteoclasts and illustrates the severity of bone erosion, while toluidin blue is used to detect cartilage proteoglycan loss. The stained samples were randomized and coded before evaluation. Histological evaluation was done using Osteomeasure™, an image analysis system. With the help of this software, lengths and dimensions of structures and cell numbers were assessed.

3.6 Arthritis Severity Score

To determine the severity of arthritis with a single value, we scored the features obtained by Osteomeasure™ and calculated the Arthritis Severity Score (ASS). An ASS ≥ 2 is indicative for arthritis. The maximum ASS of 9 indicates massive erosive arthritis.

Histomorphometric features	**Measurement**	**Points**
Inflammatory area	≥ 0,1 mm^2	1
	≥ 0,5 mm^2	2
	≥ 1 mm^2	3
Erosion area	≥ 0,01 mm^2	1
	≥ 0,05 mm^2	2
	≥ 0,1 mm^2	3
Number of osteoclasts	1	1
	2-5	2
	> 5	3

Table 3.6. Arthritis Severity Score.

3.7 Statistical analysis

Statistical analysis was performed using Graphpad Prism 6® and Microsoft Excel®.
To compare the clinical course over 8 months, the grand means of paw swelling and grip strength were assessed. On each clinical assessment, for paw swelling a minimum of 0 (no paw swollen) and a maximum of 3 (all 4 paws maximally swollen) was possible. For grip strength, a maximum of 3 (no loss of grip strength) and a minimum of 0 (total loss of grip strength) could be reached. To compare clinical and histological signs of arthritis between the experimental groups, two-tailed Student´s t-test was used at the assumption of normal distribution and homogeneity of variances. Otherwise, Mann-Whitney-U-test was performed. Comparison of arthritis-frequency after 8 months was determined with Fisher´s exact test. At all instances, a p-value of <0.05 was considered to be statistically significant. Clinical and histological results are presented using adequate tables and figures.

4 Results

4.1 Clinical course of arthritis

In line with previous literature, first clinical signs of pristane-induced arthritis arose after three months following pristane injection. Throughout all groups, paw swelling and loss of grip strength most commonly affected the hind paws.

Regarding the onset and course of the disease, the PIL-group was affected the most: it showed the earliest onset of symptoms (week 14) and the most severe course with a constant increase of paw swelling and loss of grip strength. The most rapid progression of disease was seen between the 4^{th} and 6^{th} month after disease induction (Figure 4.1.1). After 4 months (week 18) 24%, after 6 months (week 26) 52%, and after 8 months (week 32) 62% of PIL-mice developed arthritis, which was defined as an occurrence of both paw swelling and loss of grip strength.

Mice treated with monthly injections of Treg showed a milder course of the disease: After 4 months (week 18) 12%, after 6 months (week 26) 17% and after 8 months (week 32) also only 17% presented with arthritis. By analyzing the mean of the clinical evaluations throughout the experiment, we can see that the monthly injection of Treg significantly decreased the severity of the disease: Mice from the Treg-rep group presented with a higher grip strength (2.964 ± 0.024 vs. 2.732 ± 0.063, $p<0.01$) and less paw swelling (0.044 ± 0.020 vs. 0.360 ± 0.069 , $p < 0.01$) compared to the PIL-group (Figure 4.1.2). Further, the mean onset of both paw swelling and loss of grip strength was delayed in Treg rep.

The Treg boost shortly after disease induction did not significantly ameliorate the clinical course over 8 months (mean paw swelling 0.334 ± 0.045 vs. 0.3603 ± 0.068 and mean loss of grip strength 2.756 ± 0.052 vs. 2.732 ± 0.063) (Figure 4.1.2). Nonetheless, it seemed to have a retarding effect of the onset, seen in a delayed loss of grip strength.

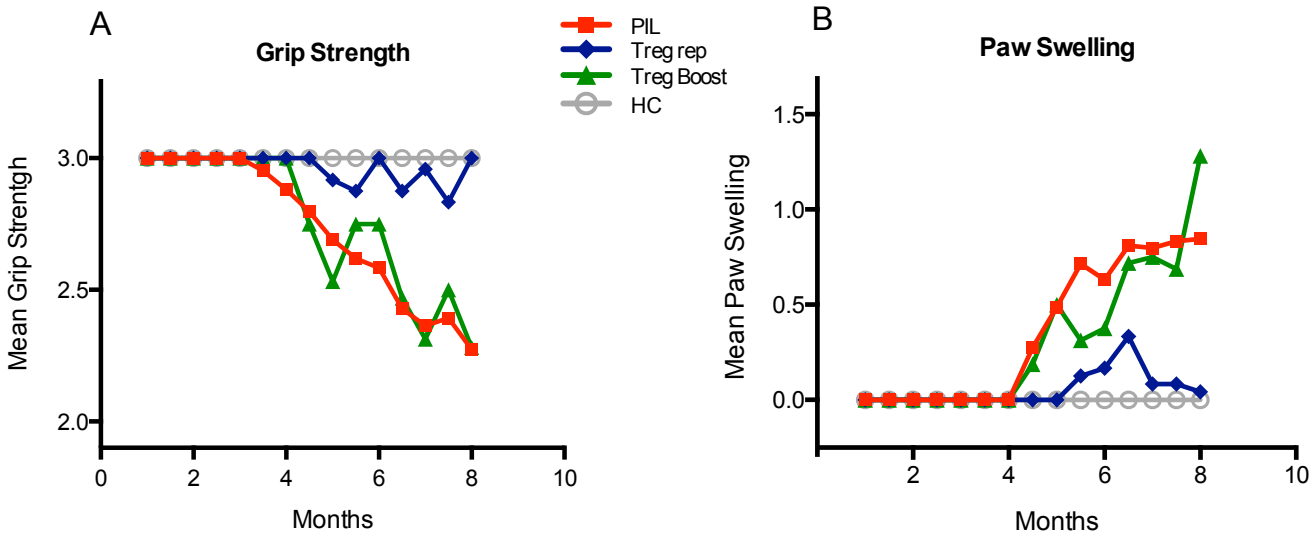

Figure 4.1.1. Clinical course of arthritis. (A) Compared to Treg-rep, PIL showed an earlier onset of loss of grip strength and a distinct more severe course. Even though Treg-boost did not ameliorate disease severity, it could delay its onset in terms of loss of grip strength. (B) Treg-rep also ameliorated disease severity in regards to paw swelling and showed a delayed onset.

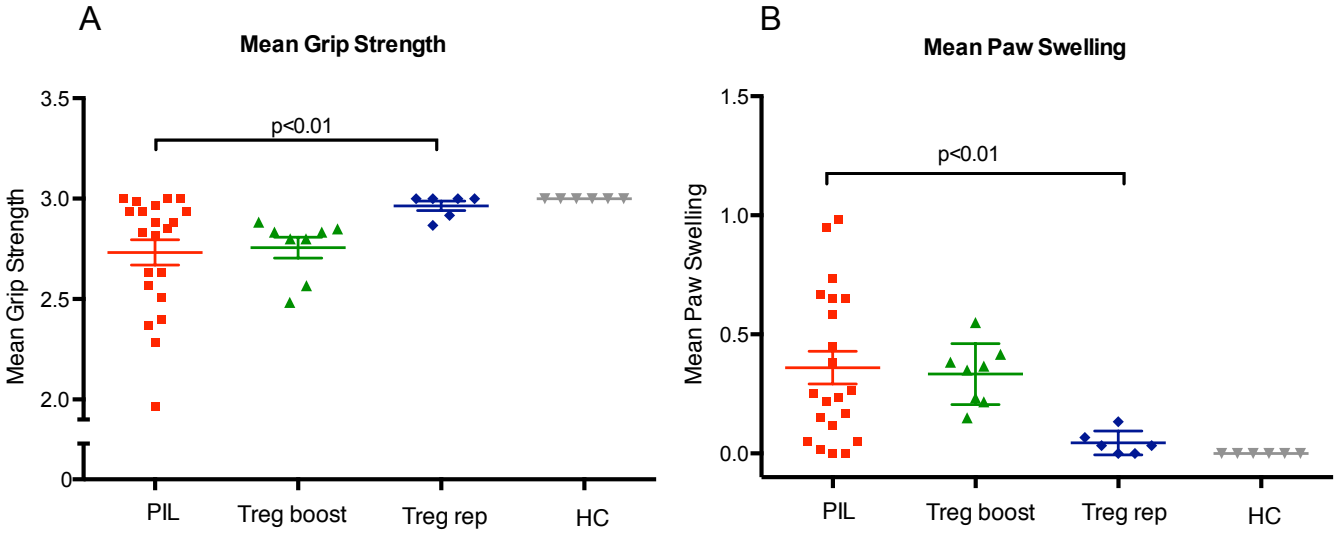

Figure 4.1.2. Mean paw swelling and grip strength over 8 months. To compare the clinical course over 8 months, the means of the mean paw swelling at each time point was assessed and compared between the different groups. The monthly injection of Treg (Treg-rep) significantly decreased clinical signs of arthritis compared to PIL: (A) Mean grip strength: 2.732 ± 0.063 vs. 2.964 ± 0.024, $p<0.01$. (B) Mean paw swelling: 0.360 ± 0.069 vs. 0.044 ± 0.020, $p<0.01$. The Treg-boost did not show a statistical difference in clinical disease severity compared to PIL.

Regarding the amount of arthritis episodes, 24% of PIL mice and 67% of Treg-rep mice never had an event of arthritis. In mice, in which joint involvement was detected at least once, the Treg-group showed less episodes compared to the PIL-group: in Treg-rep a maximum of 5 episodes was detected in 33%; no animal developed arthritis more than 5 times. In PIL-mice, 42% showed joint involvement in a maximum of 5 clinical evaluations, and 33% in more than 5 instances. The Treg-boost did not prevent the outbreak of arthritis; all animals developed disease episodes, with the majority of mice (63%) presenting 5 times or less with clinical symptoms (Figure 4.1.3). Throughout all groups, mice that presented with arthritis in over 5 clinical assessments also showed the most severe histological picture.

50% of the affected mice from the PIL-group presented with a relapsing course, whereas the other part of animals had a chronic course, characterized by the presence of clinical arthritis at each assessment point since the first disease episode. All affected mice from the Treg-group showed a relapsing course. In the Treg-boost group, 63% had a chronic course and 37% relapsing episodes.

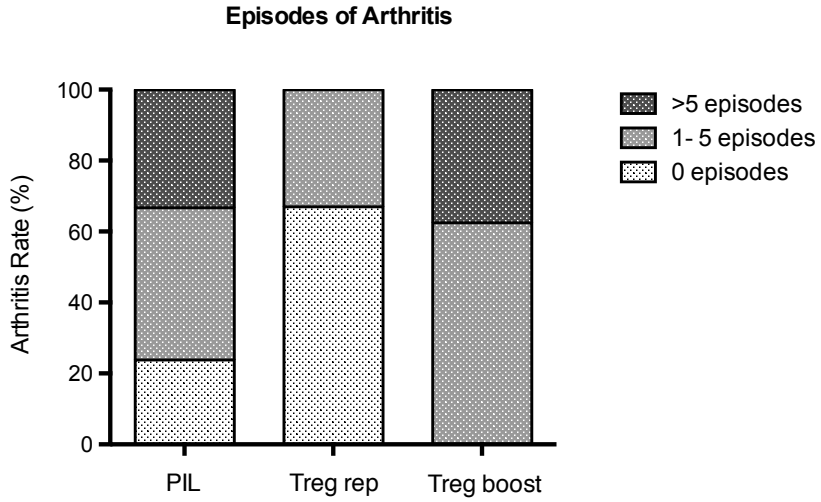

Figure 4.1.3. Arthritis episodes within the different groups. Comparing the episodes of clinically assessed arthritis, we see that most mice from the Treg-rep group did not present with arthritis at any point in the clinical evaluation. Mice that developed arthritis in this group, only showed a maximum of 5 arthritis episodes. In the PIL- and Treg-boost group the majority of mice exhibited 1 to 5 episodes, while also a significant amount presented with over 5 episodes.

4.2 Histological analysis

Analysis with Osteomeasure® software showed that 62% of PIL mice and 75% of the Treg-boost group had erosive arthritis after 8 months (Fisher´s exact: n.s.). In the Treg-rep group only 2 out of 6 (33%) showed histological signs of erosive arthritis (Fisher´s exact: n.s.).
Regarding the detailed analysis of the histological parameters (inflammatory area, erosive area, number of osteoclasts, cartilage degradation), the monthly injection of Treg significantly reduced all parameters compared to PIL. In Table 4.2.1 a comparison of the groups including the rates of significance is listed. We further calculated the Arthritis Severity Score (ASS), to assess and compare disease severity among the different experimental groups with just one single value. Since mice from the Treg-rep group had significantly milder joint involvement than PIL-mice, also the ASS was significantly reduced (4.810 ± 0.800 vs. 2.167 ± 0.946, p=0.05).

Mean	PIL	Treg-rep	Treg-boost	Significance level
Inflammatory area (mm^2)	0.688 ± 0.113	0.188 ± 0.0574		p < 0.001
			0.598 ± 0.082	p = n.s.
Erosive area (mm^2)	0.069 ± 0.017	0.011 ± 0.009		p = 0.006
			0.023 ± 0.006	p = 0.02
Number of osteoclasts	9.143 ± 1.999	2.000 ± 1.125		p = 0.005
			6.750 ± 1.800	p = n.s.
Cartilage degradation (mm^2/mm^2)	0.187 ± 0.033	0.059 ± 0.004		p = 0.001
			0.228 ± 0.041	p = n.s.

Table 4.2.1. Comparison of histological parameters. The injection of either the Treg-boost after disease induction or the monthly dose of 10^6 Treg resulted in a clear trend towards a milder grade of arthritis observed throughout all parameters, with the exeption being cartilage degradation in the Treg-boost group

Looking at mice that received the single Treg-boost at the time of disease induction, a significant difference was seen in the erosive area when compared to PIL-mice (0.023±0.006 vs. 0.069±0.017mm2, p=0.02). Area of inflammation, number of osteoclasts and cartilage degradation did not differ significantly (Figure 4.2.1). The ASS further did not differ significantly (4.810 ± 0.800 vs. 5.125 ± 0.693, n.s.).

The finding that the erosive area was significantly reduced in the Treg-boost group, led to the assumption that the single injection of $5x10^6$ Treg caused a retardation in the progression of the disease; although there was already notable destruction to the cartilage, there was no such damage observed on the bone. To gauge the extent of this retardation, we compared the Treg-boost group to PIL-mice that were sacrificed 6 months after disease induction. We here saw a similar histological picture in both groups: Among all the histological parameters evaluated, no significant variation was observed between the two groups.

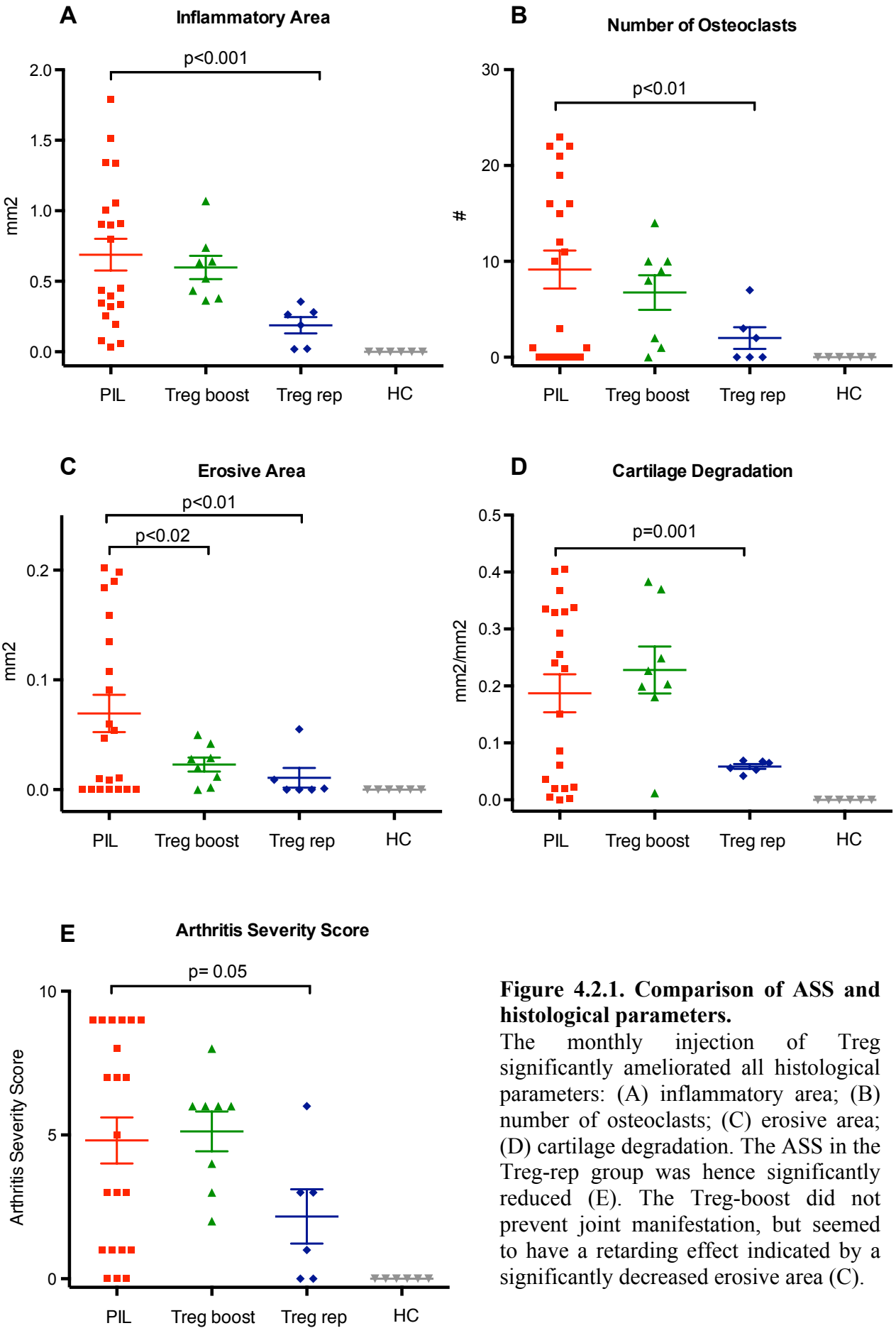

Figure 4.2.1. Comparison of ASS and histological parameters.
The monthly injection of Treg significantly ameliorated all histological parameters: (A) inflammatory area; (B) number of osteoclasts; (C) erosive area; (D) cartilage degradation. The ASS in the Treg-rep group was hence significantly reduced (E). The Treg-boost did not prevent joint manifestation, but seemed to have a retarding effect indicated by a significantly decreased erosive area (C).

When looking only at animals with histologically proven erosive arthritis, PIL-mice exhibited a 3-fold increase in severity and pronouncement across the spectrum of assessed parameters as compared to Treg-rep mice. Statistical significance was reached in the parameters 'infammatory area' and 'cartilage degradation', while other parameters did not meet the standards of statistical significance, assumably due to the small number of mice that were affected from the Treg-group.

Regarding the Treg-boost group, there also was a clear trend towards a milder grade of arthritis observed throughout all parameters (Fig.4.2.2).

	affected PIL	**affected Treg-rep**	**affected Treg-boost**	**Significance level**
Inflammatory area (mm^2)	0.981 ± 0.121	0.227 ± 0.038		p = 0.03
			0.664 ± 0.096	p = n.s.
Erosive area (mm^2)	0.112 ± 0.020	0.032 ± 0.230		p = n.s
			0.030 ± 0.006	p = 0.01
Number of osteoclasts	14.77 ± 1.96	5.0 ± 2.0		p = n.s.
			8.833 ± 1.6	p = n.s
Cartilage degradation (mm^2/mm^2)	0.276 ± 0.032	0.060 ± 0.004		p < 0.05
			0.248 ± 0.036	p = n.s.

Table 4.2.2. Comparison of histological features of affected mice. Compared to affected PIL-mice, affected mice from the Treg-rep group as well as from the Treg-boost group showed a less severe histological picture of arthritis.

4.3 Correlation of clinical and histological findings

Comparing the clinical observations with the histological data, we found that 86% of mice that presented with clinical arthritis also had erosive arthritis in histology when analyzed after 8 months. Further, 82% of mice with histological arthritis had arthritis in the clinical assessment.

The two clinical features, paw swelling and loss of grip strength, correlated well with each other ($r=0.928$, $p<0.0001$) and also correlated with all histological parameters and the Arthritis Severity Score (Figure 4.3.1, Fig. 4.3.2).

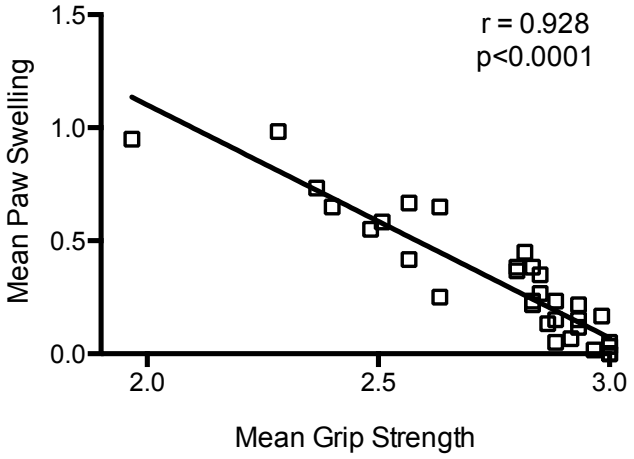

Figure 4.3.1. Correlation of clinical parameters. Throughout the experimental groups, both clinical parameters, paw swelling and grip strength, correlated well with each other ($r=0.0928$, $p<0.0001$).

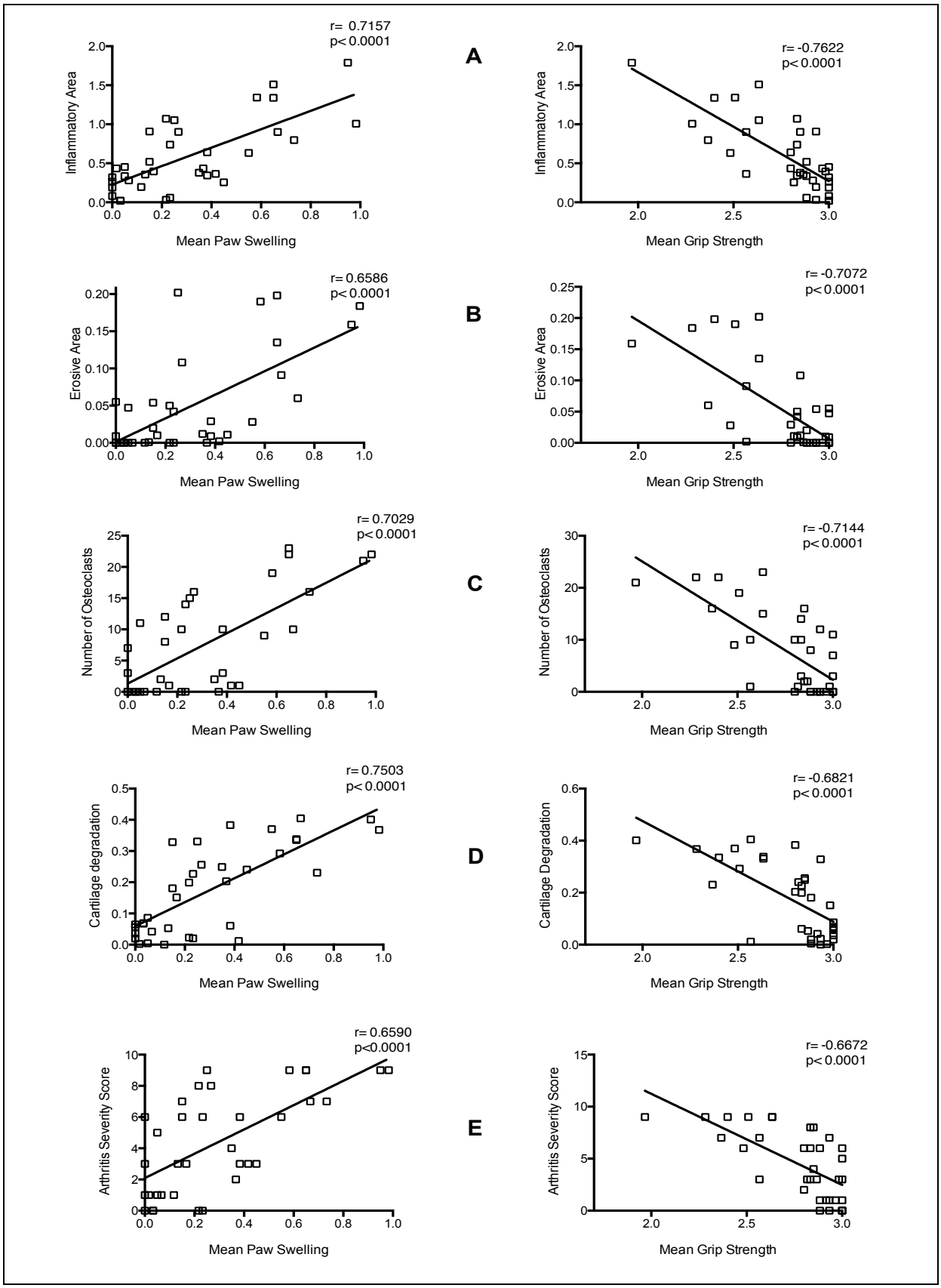

Figure 4.3.2. Correlation of clinical and histological parameters. Clinical features of arthritis (paw swelling and grip strength) correlated with histological parameters obtained by

Osteomeasure®: (A) inflammatory Area, (B) erosive area, (C) number of osteoclasts, (D) cartilage degradation, and (E) ASS.

In conclusion, the monthly injection of regulatory T-cells was able to ameliorate both the clinical and histological course of arthritis. The single injection of $5x10^6$ Treg did not prevent the manifestation of joint involvement, but seemed to have a retarding effect indicated by a retardation in the parameter 'loss of grip srength' and by a significantly less erosive area. Clinical as well as histological parameters correlated well with each other.

4.4 Pictures of histological cuts

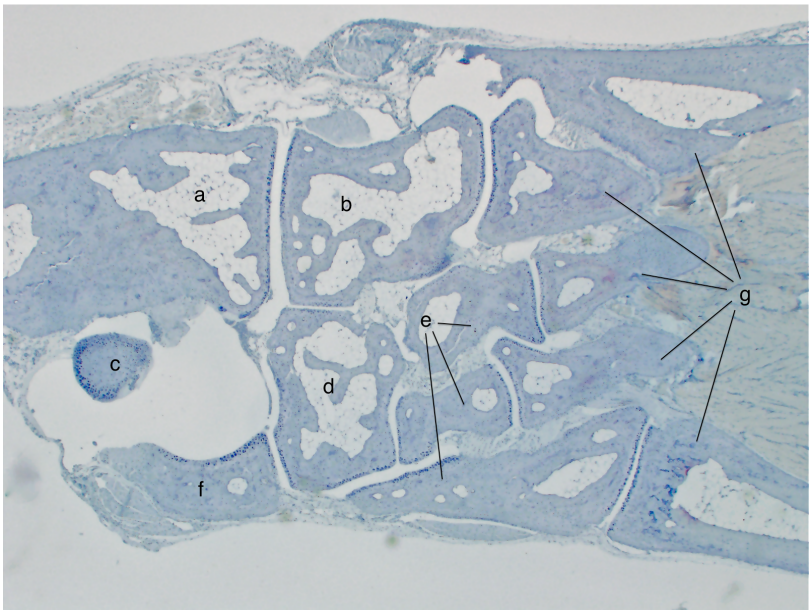

Figure 4.4.1. Overview of the hind paw from a healthy mouse. In the histological evaluation of the hind paws only the bones of the tarsus and the tissue around these bones were analyzed. (a) calcaneus, (b) cuboid bone, (c) talar bone, (d) navicular bone (e) cuneiform bones I-III, (f) fibula, (g) metatarsal bones I-V.

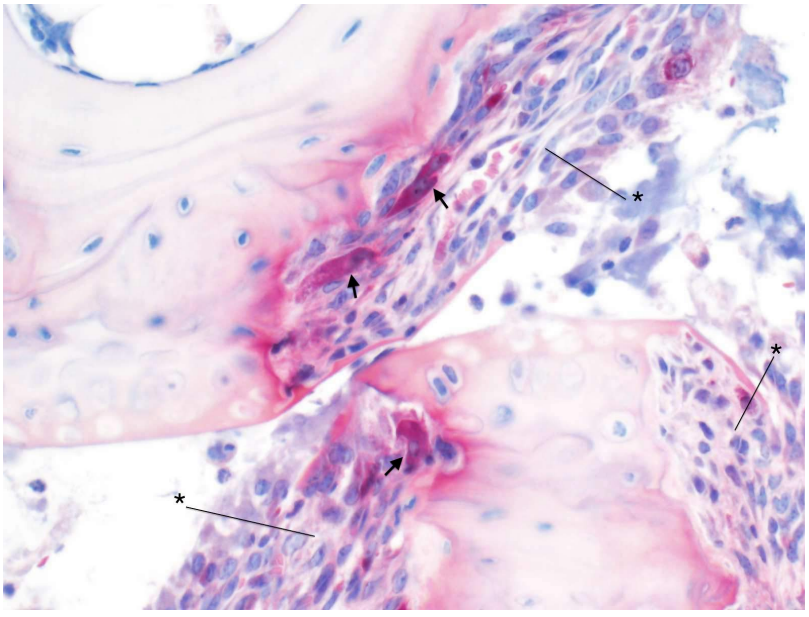

Figure 4.4.2. Site of erosive bone destruction in PIL. Erosion in PIL arthritis is superficial and rarely affects both cortical layers of the bone. Activated osteoclasts (arrows), that are embedded in inflammatory tissue (*) degrade the bone surface.

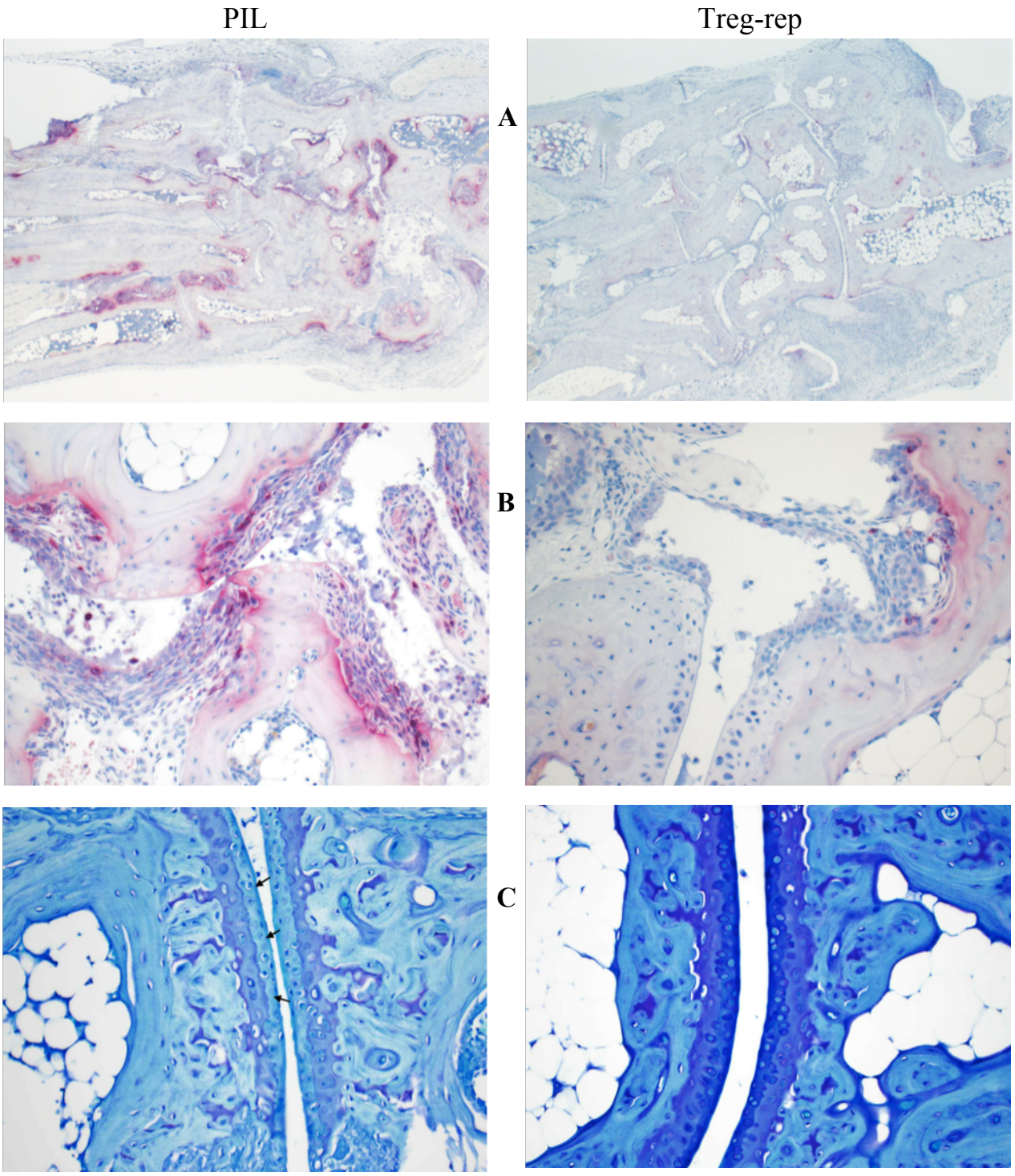

Figure 4.4.3. Comparison of histological staining of the PIL- and Treg-rep group. (A) and (B): TRAP-staining revealed the extend of inflammation and osteoclastic activity. In the PIL-group severe bone destruction and inflammatory infiltrate was seen throughout the tarsus. In the Treg-rep group, affected animals showed a less severe histological picture with only mild erosion and little inflammatory area. (C) Staining with toulidine blue was used to asses the severity of cartilage degradation. Loss of proteoglycan from tissue resulted in destaining of the cartilage (arrows). The PIL-group presented with a more drastic loss of cartilage, whereas mice treated with Treg had only mild cartilage degradation.

5 Discussion

Recent research has proven regulatory T-cells to play a major role in the immune homeostasis and the development of autoimmune diseases. Not only in animal models, but also in the human body, experiments showed that a lack of Treg leads to a higher susceptiblity of a diversity of autoimmune diseases; for instance, a lack of Treg in mice (scurfy mice) results in a fatal multi-organ autoimmune disease with many resemblances to human SLE.[114] In the human body, decreased Treg numbers and function were associated with diseases such as SLE and inflammatory bowel disease.[68,115]

It hence is not far-fetched that research now focuses on Treg as a potential therapeutic approach to treating diseases. So far, multiple animal studies have demonstrated that Treg can be used to treat auto-inflammatory diseases including autoimmune gastritis, type 1 diabetes and inflammatory bowel disease.[90,91] Promising results in the fields of transplant rejections and in severe refractory graft versus host disease (GvHD) were the first in-human experiments that verified Treg as a potential immunosuppressive therapy in the human body.[94] In terms of SLE, clinical trials addressing the adoptive transfer of Treg within humans have not been performed yet; animal testing continues to be crucial in evaluating Treg therapy.

In line with the mentioned recent work, our study underscores the potential of regulatory T-cells to reduce the severity of autoimmune diseases. We assessed the influence of Treg on joint involvement in the model of Pristane Induced Lupus. As described in a prior conducted study from our research group, this model has proven as a good model to investigate joint involvement in human SLE.[93] Today, joint invovlement is not primarily in the focus of lupus-research, since also other, organ involving and potentially life threatening conditions have to be dealt with. However, up to 95% of patients report joint involvement throughout their patient career.[52] Additionally, patient-bases surveys revealed joint involvement as a major burden leading to functional impairment and significant obstacles in their daily lives.[50,51]

With this study we could demonstrate that the addition of regulatory T-cells can positively influence the severity of joint involvement. A monthly injection of 10^6 regulatory T-cells ameliorated the clinical course of arthritis by retarding its onset by several weeks and also by a less severe course of the disease. If it also could prevent the outbreak of the disease is hard to estimate, since only about 60% of animals develop arthritis in this mouse model. Also in the histological analysis of the hind paws, we saw that mice treated with 10^6 Treg monthly had significantly less severe joint involvement compared to PIL-mice.

Mice that received a single $5x10^6$ Treg boost at the time of disease induction did not show a milder clinical course, apart from a slight retardation in the clinical parameter 'loss of grip strength'. Both mean paw swelling and grip strength did not differ significantly from the PIL-group. Interestingly, in the histology of the hind paws, we saw that such a boost could slow the histological disease progression by several weeks: At the end of the experiment mice from the Treg-boost group indeed had the same amount of cartilage destruction as PIL-mice, but had significantly less damage to the bone. The average life span of lymphocytes is few weeks to months. We hence suggest, that even though the Treg-boost at the time of disease induction could not prevent the outbreak or severity of erosive arthritis, it was able to retard its onset for several weeks due to the suppression of autoreactive lymphocytes, that play a major role in the disease development.

In order to facilitate a meaninful statistical approach, this research extrapolated from limited populations and thus assumed the conditions necessary for the application of statistical analysis. Mice, as trial species, allow for such assumptions due to their high level of genetic homogeinity as one would not assume large levels of variation among the population. This methodology aimed to produce results that illustrate and quantify trends potentially seen in larger population groups. In general, as animal trial populations can be limited, there are restrictions to statitical analysis and the interpretation of the results. Due to factors such as greater homoscedasticity as well as normal distribution, an increased population size would allow for stronger statistics. An increase in population size further midigates the risks associated with fatalities trhoughout the course of experminentation, inherent to a mouse model approach.

With this study, however, we could make another vital contribution on the beneficial transfer of regulatory T-cells in autoimmune diseases. Further work from our research group also indicates that Treg are capable to ameliorate inner organ involvement in PIL.[106] However, before Treg can actually be implemented as a therapeutic agent, research has to resolve a number of questions concerning topics such as Treg acquisition, purity, dosage and safety. Our experiment evinces, that dosage and the proper time of Treg application are critical aspects that need be considered. Previous literature suggested doses with up to $1x10^9$ Treg per infusion to achieve effective immunosuppression in animal-testing for transplant rejection.[116] Weigert *et al.* could prolong the interval of remission in (NZBxNZW) F1 mice with a single adoptive transfer of $1.5x10^6$ Treg.[117] Studies like these demonstrate, that a 'perfect' dosage

may need to be established for every disease and disease stage itself. In terms of acquisition, several approaches have been tested for isolating and cultivating the ideally potent and efficient regulatory T-cell. Studies suggested that iTreg are more stable in the inflammatory environment and also show more potency than nTreg, making strategies expand iTreg a more attractive one.[90]

Issues like these demonstrate that a comprehensive amount of research activity has to be conducted until Treg can actually be used as a 'weapon against autoimmunity'. Through the results and methods developed in this study, we hope to have contributed to the process of reaching this ambitious goal.

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List of abbreviations

ACLE	Acute cutaneous lupus erythematosus
ACR	American College of Rheumatology
ANA	Anti-nuclear antibody
anti-Sm	anti-Smith
APC	Antigen-presenting cells
BALB/C	“Bagg albino”
BILAG	British Isles Lupus Assessment Group Index
BLys	B-Lymphocyte stimulator
C57BL6	Crossing 57 Black Little Sub strain 6
CCLE	Chronic cutaneous lupus erythematosus
CD	Cluster of differentiation
CMV	Cytomegalic Virus
CNS	Central nervous system
CTLA	Cytotoxic T-lymphocyte-associated antigen
DC	Dentritic cell
DILE	Drug-induced lupus erythematosus
dsDNA	Double stranded deoxyribonucleic acid
ECLAM	Euopean Consensus Lupus Activity Measure
EBV	Ebstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EULAR	European League Against Rheumatism
Foxp3	forkhead box protein 3
GITR	Glucocorticoid-induced TNF-receptor
GVHD	Graft-versus host disease
HC	Healthy control
HDAC	histone deacetylase
i.p.	Intraperitoneal
IBD	Inflammatory bowel disease
ICAM	Intracellular adhesion molecule
IDO	Indoleamine 2,3-diosygenase
IFN	Interferon

Ig	Immunoglobulin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome
IL	Interleukin
iTreg	Inducible regulatory T-cell
LAG	Lymphocyte activated gene
LDIQ	Lupus Damage Index Questionnaire
MACS	Magnetic Cell Separation
MCP	Metacarpal phalangeal
MCTD	Mixed connective tissue disease
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MRI	Magnetic resonance imaging
MTX	Methotrexate
n.s.	not significant
NKT	Natural Killer-cell
NRP	Neutropilin
NSAIDs	non steroidal anti inflammatory drugs
nTreg	natural regulatory T-cell
NTx	neonatal thymectomy
NZB/W	New Zealand Black/White
PBS	Phosphate buffered saline
PIL	Pristane induced lupus
PTPN22	protein tyrosine phosphatase 22
RA	Rheumatoid Arthritis
SCLE	Sub-acute cutaneous lupus erythematosus
SDI	SLICC/ACR Damage Index
SF-36	Short-Form-36
SIS	SLE Activity Index Score
SLAM	Systemic Lupus Activity Measure
SLE	Systemic Lupus erythematosus
SLEDAI	SLE Disease Activity Index
SLEQoL	Systemic Lupus Erythematosus Quality of Life
SLICC	Systemic Lupus International Collaborating Clinic
TB	Toluidine blue

TCR	T-cell receptor
TGF β	Transforming Growth Factor β
Th3	T-helper 3
TNF α	Tumor necrosis factor α
Tr1	T-regulatory 1
TRAP	Tartrate resistant acid phosphatase
Treg	Regulatory T-cell
UV	Ultra-violet

Attachment

VIENNA LUPUS GROUP

MEDICAL UNIVERSITY OF VIENNA

IN VITRO INDUCED REGULATORY T CELLS CAN AMELIORATE THE SEVERITY OF LUPUS ARTHRITIS

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Abstract.

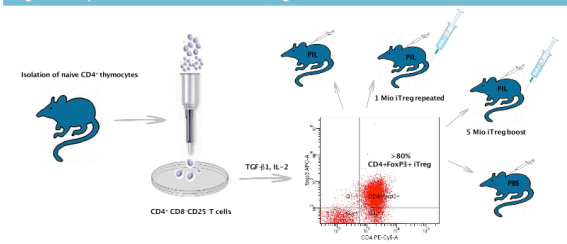
Introduction.

Arthritis is often reported as a first symptom of systemic lupus erythematosus (SLE) and is seen in the majority of patients over the course of the disease. Even though joint involvement is a non-life-threatening condition, patient surveys reveal arthritis as a major burden of SLE patients leading to impairment and daily life hurdles.
We herein investigate the therapeutic effects of regulatory T cells (Treg) in the murine model of pristane induced lupus in regard to joint involvement.

Methods.

Mice were injected i.p. with 0.5ml of pristane or PBS as control and killed after 8 months. Naïve $CD4^+$ thymocytes were cultured under Treg-inducing conditions and tested for $CD4^+Foxp3^+$ expression by FACS. Cell suspensions with >80% purity for $CD4^+FoxP3^+$ iTreg were injected intravenously either once at start of experiments (iTreg-boost) or monthly (iTreg-rep) over the course of the experiment. Animals were monitored for clinical signs of arthritis: Paw swelling and grip strength were assessed every 2 weeks using semi-quantitative scores. Histological features of arthritis were quantified by Osteomeasure Software, an image analysis system.

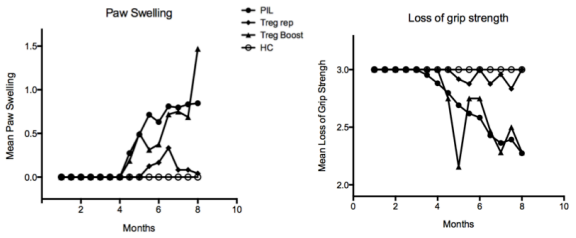
Figure 1. Experimental course of PIL & iTreg Induction.



Results.

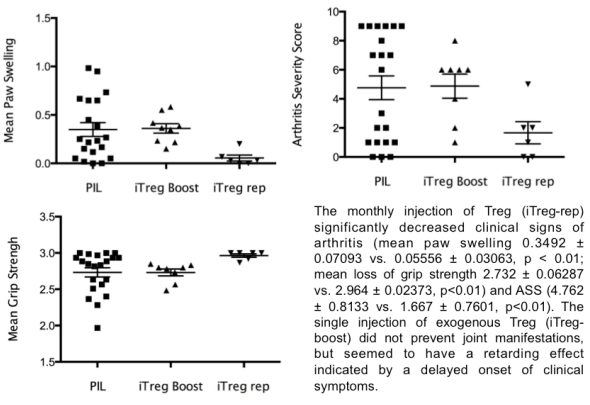
Clinically, PIL group developed distinct signs of arthritis after 14 weeks. 52% out of the PIL group and 36% of the Treg-treated groups developed at least one episode of arthritis within 8 months of observation.

Figure 2. Treg retard the onset of arthritis.



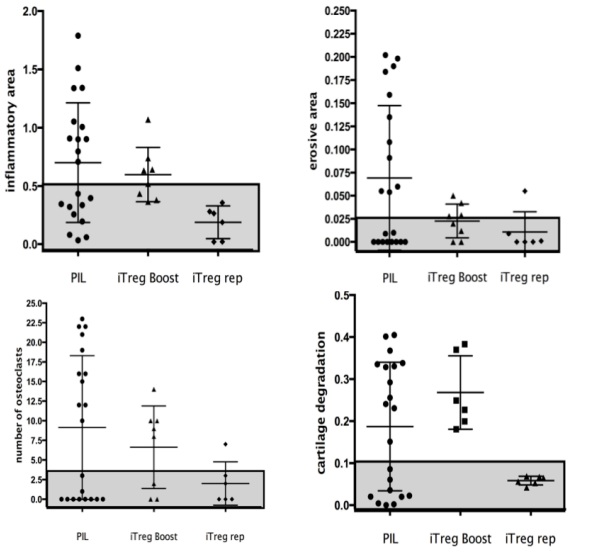
Regarding the onset and course of the disease, PIL-group seems to be affected the most: compared to iTreg-rep, PIL showed an earlier onset and a more severe course with a constant increase in paw swelling and decrease in grip strength.

Figure 3. Treg reduce the severity of arthritis.



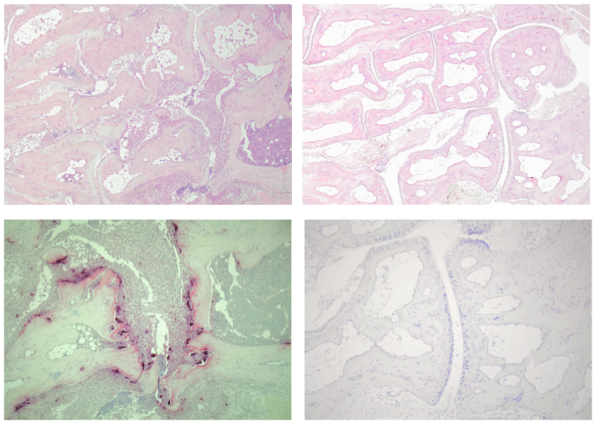
The monthly injection of Treg (iTreg-rep) significantly decreased clinical signs of arthritis (mean paw swelling 0.3492 ± 0.07093 vs. 0.05556 ± 0.03063, p < 0.01; mean loss of grip strength 2.732 ± 0.06287 vs. 2.964 ± 0.02373, p<0.01) and ASS (4.762 ± 0.8133 vs. 1.667 ± 0.7601, p<0.01). The single injection of exogenous Treg (iTreg-boost) did not prevent joint manifestations, but seemed to have a retarding effect indicated by a delayed onset of clinical symptoms.

Figure 4. Diminished histological signs of arthritis due to Treg.



The monthly injection of Treg (iTreg-rep) significantly ameliorated all histological parameters (inflammatory area 0.7007 ± 0.1120 vs. 0.1882 ± 0.05742 mm2, p<0.01; erosive area 0.06930 ± 0.01707 vs. 0.01083 ± 0.008950 mm2, p<0.01; number of osteoclasts 9.143 ± 1.999 vs. 2.000 ± 1.125, p<0.01; cartilage degradation 0.1871 ± 0.03337 vs. 0.05857 ± 0.004209, p<0.01). Out of 6 mice treated, 4 (66%) did not show any signs of arthritis at all.
The single injection of exogenous Treg (iTreg-boost) did not prevent joint manifestations, but seemed to have a retarding effect indicated by a decrease in the size of the mean erosive area (mean 0.069±0.017 vs. 0.022±0.006 mm2, p<0.05); the area of inflammation, number of osteoclasts and cartilage degradation did not differ from PIL group.

Figure 5. Exemplary comparison of histological sections of PIL and Treg-rep.



Conclusion

REPEATED INJECTIONS OF EXOGENOUSLY INDUCED TREG PREVENT DISTINCT CLINICAL SIGNS OF ARTHRITIS AND SIGNIFICANTLY DECREASE LUPUS ARTHRITIS IN PIL MICE. A SINGLE BOOST OF TREG EVEN WHEN APPLIED BEFORE THE CLINICAL ONSET CANNOT DECREASE ALL PARAMETERS OF ARTHRITIS, BUT APPEARS TO RETARD ONSET OF SYMPTOMS AND EROSIVE PROGRESSION.
THUS, TREG HAVE SIGNIFICANT EFFECTS ON LUPUS ARTHRITIS WHEN APPLIED REPEATEDLY, WHICH MAY HAVE CONSEQUENCE FOR FUTURE THERAPEUTIC CONSIDERATIONS.