

# **Early upregulation of Socs1 contributes to antigen-specific tolerance in collagen-induced arthritis**

Master thesis in Medicine

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## ABSTRACT

### INTRODUCTION:

To study tolerogenic immunological mechanisms we use lentiviral based gene therapy in collagen-induced arthritis. In our tolerogenic model, collagen type II expression is enhanced on MHC class II on all types of antigen-presenting cells. After this treatment only 5% of mice develop arthritis at the same time as >95 % of control mice do. This study's focus is before immunisation and days 3 and 5 after.

### METHOD:

Gene expression analysis in draining lymph nodes was performed using an immune card array, where the mRNA expression of 96 inflammation related genes was determined by quantitative PCR. Differences in relative quantification was investigated using multivariate analyses OPLS-DA and PLS. The presence of regulatory T cells in spleen and draining lymph nodes was determined by CD4 and Foxp3 expression using flow cytometry.

### RESULTS:

At day 3 mRNA expression of both *Socs1* and *Il10* was positively while *Ifng* and *Il6* was negatively associated to tolerance. At the same time the frequency of CD4+FoxP3+ cells were significantly increased in tolerant mice.

### CONCLUSIONS:

In tolerant mice, overexpression of collagen type II on MHC II leads to upregulation of SOCS1 at day 3 after CIA induction. SOCS1 inhibits the JAK/STAT pathway.

Simultaneously, we see down-regulation of pro-inflammatory cytokines such as IFN-g and IL-6 as well as to increased levels of immunoregulatory IL-10. These findings coincide with an expansion of regulatory T cells. Thus, the arthritogenic immune response is switched off during an early stage of CIA and aborts arthritis development.

## INTRODUCTION

The immune system of a healthy individual is tolerant towards the constituents of its host's tissues. Though protected through a multitude of failsafe mechanisms this tolerance can be breached and autoimmune disease be developed. Autoimmune disease is characterised by an immune response towards self-antigens.

Rheumatoid arthritis (RA) the most common rheumatoid disease with a prevalence of 0.5 – 1%, exceeded only by osteoarthritis. During the last decades treatment of RA and other autoimmune diseases has been revolutionised with the introduction of biological treatments. Even though this has greatly improved our ability to manage disease progress there are still non-responders and immunosuppression itself is associated with severe side effects such as infections and malignancies. An intriguing future treatment would be to re-establish the self-tolerance once broken. This would treat the cause of the disease and potentially cure it.

One of the recognised self-antigens in RA is collagen type II (CII). CII makes up 85-90% of the collagen content of articular cartilage and is expressed exclusively in joints and the vitreous humour. In this study we use collagen-induced arthritis (CIA), which is a well-established mouse model of RA. CIA was first described by Trentham et al (1) in 1977.

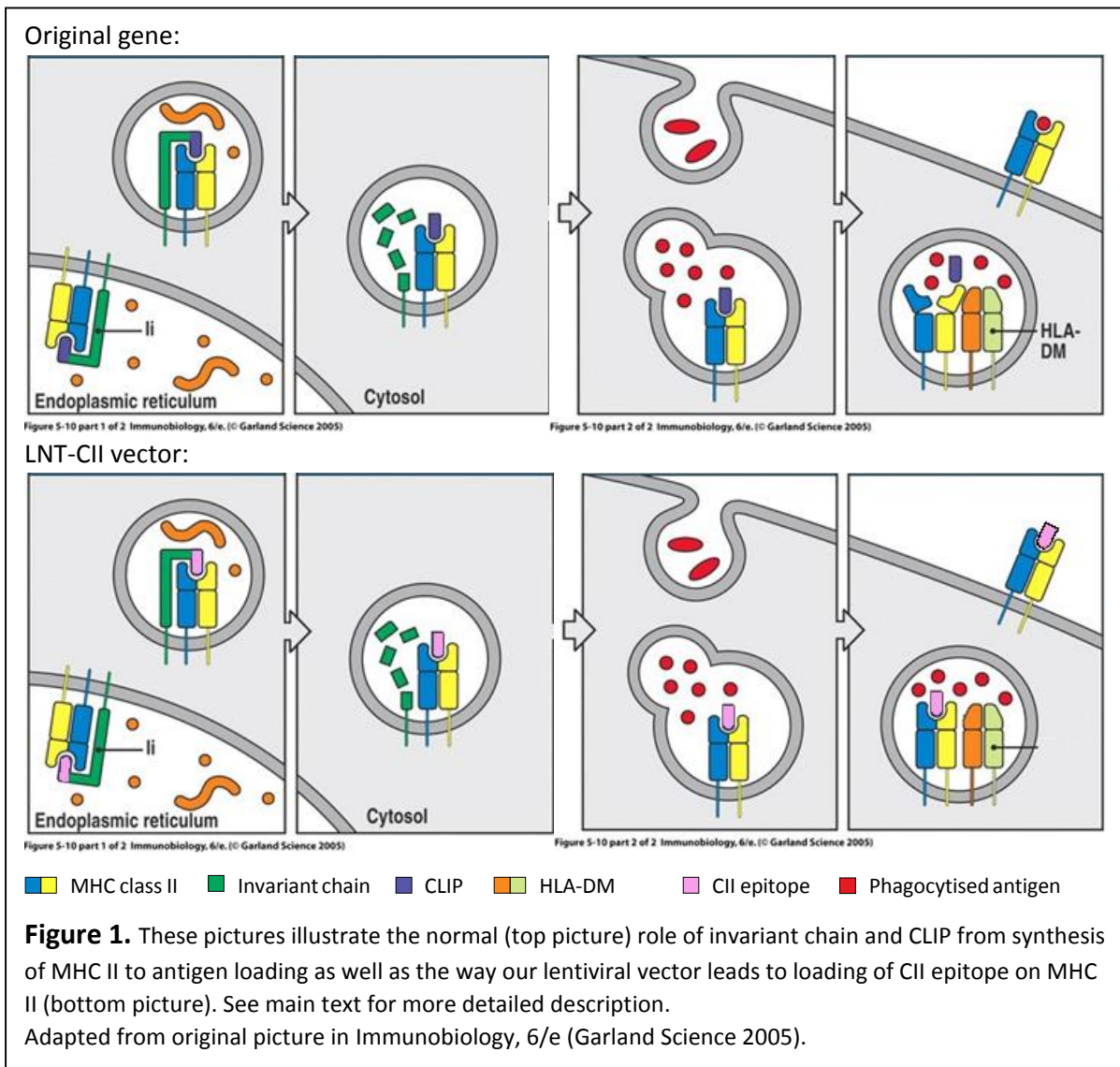
Typically mice are immunised through injection of a CII and complete Freund's adjuvant and boosted with an injection of collagen type II and incomplete Freund's adjuvant 3-5 weeks later. Clinical symptoms of arthritis can be observed within 4 to 5 weeks after immunisation. Histologically, inflamed joints of CIA and RA resemble one another, with synovial immune cell infiltration, cartilage destruction and bone erosion (2). The aetiology of RA is not fully understood, but certain MHC II haplotypes is highly associated with disease susceptibility. These haplotypes share certain sequences in the protein binding pocket (3) suggesting a common required affinity. Likewise CIA pathogenesis require certain MHC II H-2 complex

haplotypes with high affinity for CII. Serologically rheumatoid factor, anti-citrullinated protein antibodies and anti-CII antibodies have been observed in both conditions. In humans, anti-CII antibodies seems to be present particularly at onset of RA (4, 5) and are associated with a good prognosis (6).

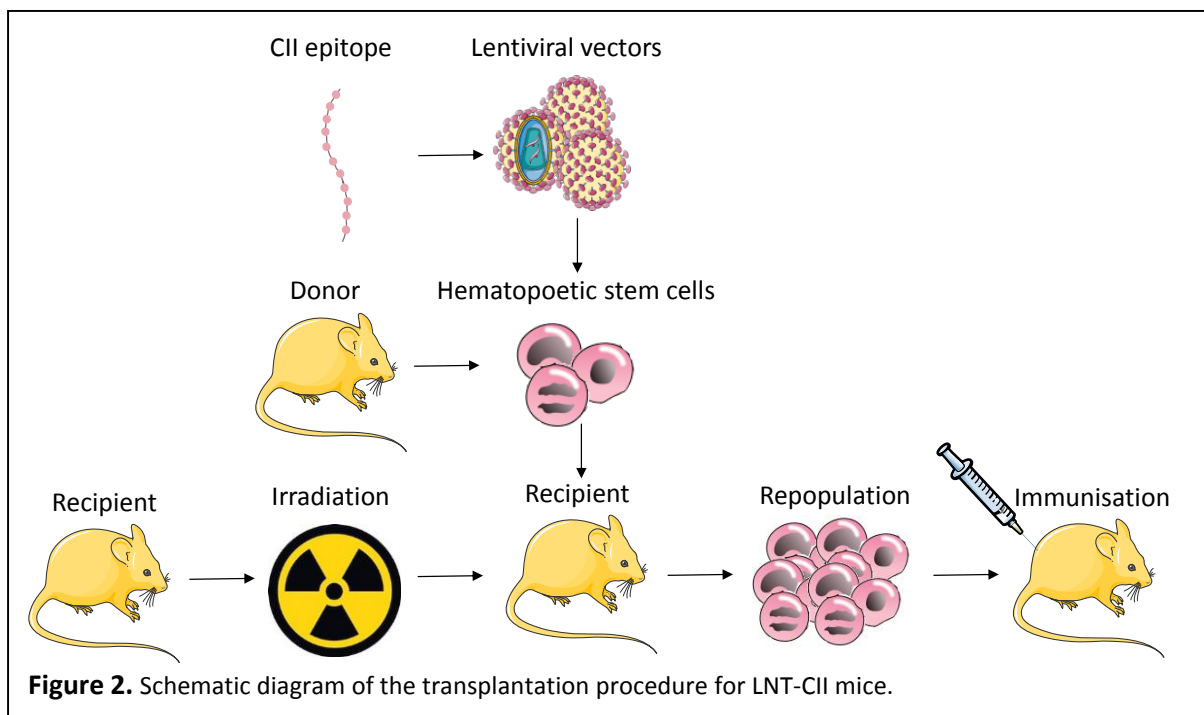
It has long been known that tolerance can be induced in mice by prophylactic treatment with soluble CII under non-inflammatory conditions (7-9). The immunodominant T cell epitope of CII in both RA and CIA is amino acids 259-270 (10-16). To further investigate the importance of different types of antigen presenting cells (APCs) in development and maintenance of CIA, Inger Gjertsson et al (17) published a report using lentiviral based gene therapy as a way to increase endogenous loading of this CII epitope on MHC II in mice. The advantages of this approach is that it 1) allows prolonged expression of the CII epitope (aa 259-270) on APCs, 2) specific APCs e.g. B cells can be targeted and 3) the epitope expression is endogenous and does not activate the APC.

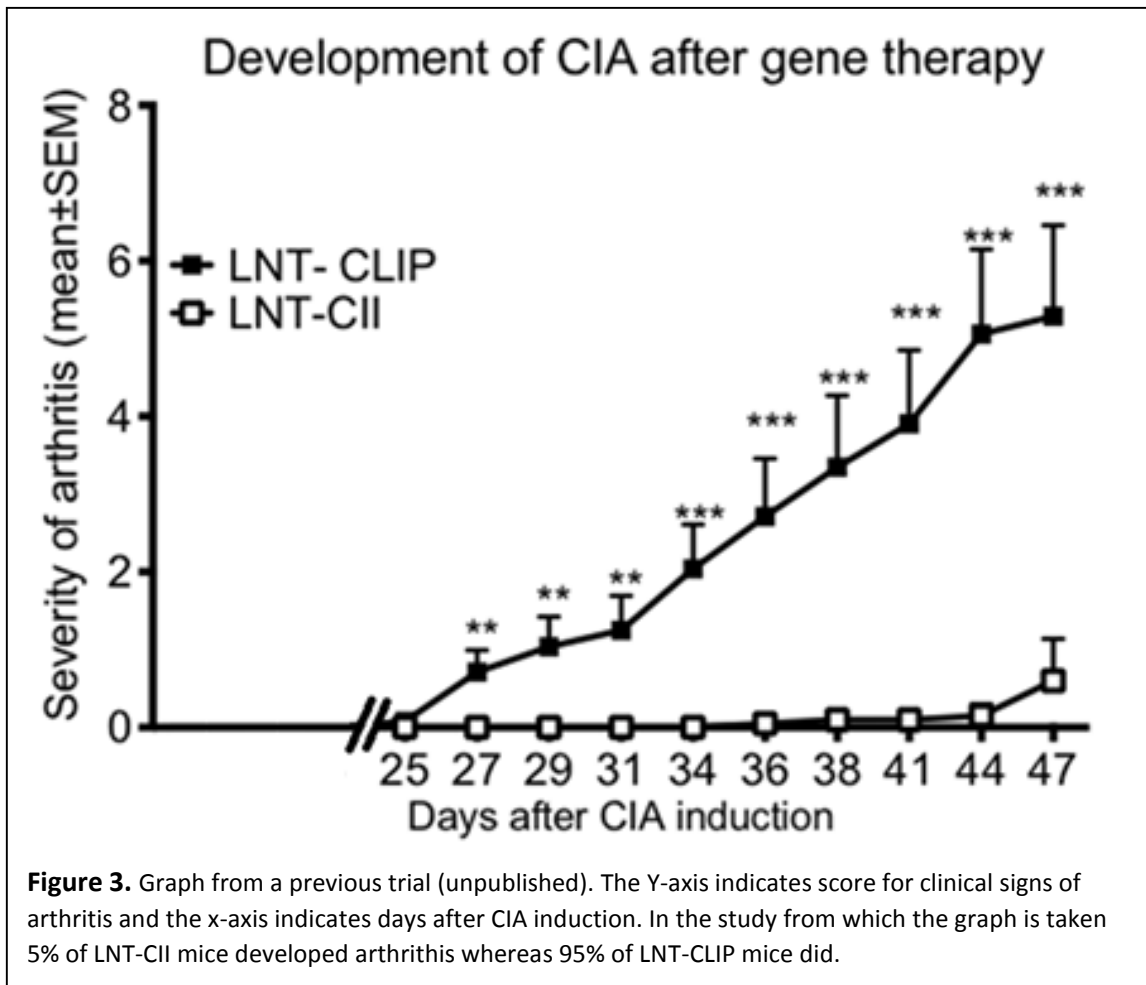
An efficient way to force expression of a peptide on MHC II is to insert it in invariant chain (18). This approach was used to construct a lentiviral vector where the expression of the CII T cell epitope (aa 259-270) was forced on MHC II by exchanging the original class II-associated invariant chain peptide (CLIP) sequence for the CII aa 259-270 sequence (LNT-CII vector). Normally, the invariant chain is cleaved and dissociated from the MHC II complex in the acidic environment of the endosome (figure 1). CLIP occupies the protein binding groove in MHC II remains bound to prevent binding of endogenous peptides. Through interactions with HLA-DM, CLIP is eventually dissociated and a phagocytised antigen is loaded on MHC II and presented to T cells on the cell surface (19). Mice susceptible to CIA carry the H2q-haplotype, which have a high affinity to CII epitope (aa 259-270). Using our

vector, this leads to inability of the CII epitope to dissociate from MHC II during loading process (figure 1). In turn, this leads to abnormally high loading of the collagen type II epitope on MHC II on all antigen presenting cells.



Since that first report, our group has performed several studies, all but one unpublished at present (20), where haematopoietic stem cells are harvested from donor mice and transduced in vitro with lentiviral particles (figure 2). Recipient mice are lethally irradiated and transplanted with transduced haematopoietic stem cells. In a previous unpublished study this treatment induced substantial antigen-specific tolerance in LNT-CII animals. Only 5% of them developed arthritis whereas 95% of LNT-CLIP animals did (figure 3). How the tolerance is mediated is not fully understood. In this previous study a 96 gene quantitative PCR array of mRNA of inflammatory genes was carried out at day 14 and day 28 after CIA induction. Results were inconclusive. To investigate the immunological events during the early phase after immunisation we now perform the 96 gene quantitative PCR array at day 0, day 3 and day 5 after CIA induction, with the aim to identify potential mediators of tolerance that could provide novel targets for tolerance induction in human RA.





### Interferon gamma and Th1/Th17 cells in arthritis

Interferon gamma (IFN- $\gamma$ ) is considered a pro-inflammatory cytokine and is the hallmark of  $T_{H1}$  cells. The main objective for  $T_{H1}$  cells is to combat intracellular bacteria and viruses.

Activation of the IFN- $\gamma$  receptor leads to JAK/STAT pathway signalling through phosphorylation of STAT1 (21). Together with IL-12, IFN- $\gamma$  stimulates naïve  $CD4^+$  cells to T-bet expression and  $T_{H1}$  differentiation. IFN- $\gamma$  is produced mainly by  $T_{H1}$  as well as natural killer cells and activates macrophages and stimulate IL-12 secretion by dendritic cells in a positive feedback loop.

Historically the view of T helper cells has been based on the concept of the dichotomy of the  $T_{H1}$  and  $T_{H2}$  cells.  $T_{H1}$  cells were believed to be responsible for the tissue damage seen in



infectious as well as autoimmune inflammation. With the description of the novel T<sub>H17</sub> subset, this perception has been reviewed during the last decade. Though T<sub>H1</sub> is still believed instrumental in initiation of autoimmune arthritis, focus has shifted towards T<sub>H17</sub> for maintaining inflammation and progressive tissue damage (22-25).

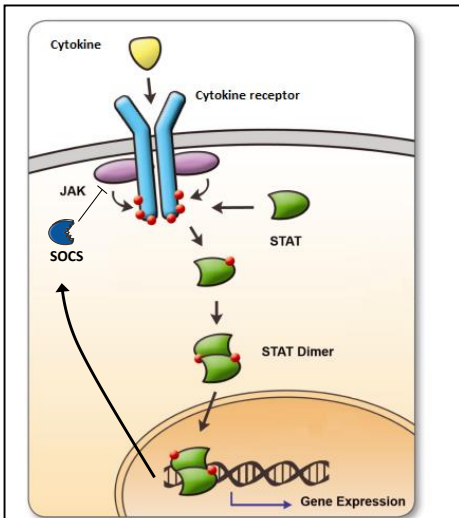
The exact role of IFN- $\gamma$  in arthritis pathogenesis is not conclusively determined. Recent research have indicated a protective role in established RA and CIA by favouring T<sub>H1</sub> development over T<sub>H17</sub> (26, 27).

### T regulatory cells and IL-10

T regeulatory cells (Treg) are characterised by the expression of the transcription factor FoxP3, surface molecule CD25 (IL-2 receptor), co-inhibitory molecule CTLA-4 and secretion of anti-inflammatory IL-10. Treg induction takes place in the thymus and in peripheral lymphoid tissue. Tregs can inhibit inflammatory responses by secretion of anti-inflammatory cytokines e.g. IL-10 and IL-35 and membrane-bound TGF- $\beta$  as well as direct cell-to-cell interactions. The latter seems to have the stronger suppressive effect (28). Stimulation of the IL-10 receptor gives rise to increased intracellular levels of phosphorylated STAT1 and STAT3.

### SOCS1 and the JAK/STAT pathway

The supressor of cytokine signalling (SOCS) family of proteins, reviewed by multiple authors (29-31), are induced by various cytokine signalling and have a regulatory function through



**Figure 4.** Schematic diagram showing the principals behind JAK/STAT pathway signalling. A cytokine binding its receptor leads to conformation changes in the receptors intracellular domain. These, in turn, allows JAKs to bind. JAKs phosphorylates the receptor allowing for STATs to bind the receptor, leading to phosphorylation of the STATs. Phosphorylated STATs dissociates from the receptor, dimerises and relocate to the nucleus to act as transcription factors. Induced transcription typically result in increased cytokine production, receptor upregulation and negative feedback through the SOCS family of proteins. Image adapted from Wikipedia (<http://en.wikipedia.org/wiki/File:Formalization-of-Jak-Stat-Pathway.png>).

negative feedback on the JAK/STAT pathway (figure 4). As with other intracellular signalling pathways, our understanding of the complex mechanics of the JAK/STAT pathway is likely crude at best. Cytokines of paradoxical effects can signal through phosphorylation of the same STAT and the different cellular responses to this signalling are likely modified by other parallel signalling pathways.

SOCS1 exerts its negative feedback by dephosphorylation of JAKs as well as targeting them for degradation by the proteasome (figure 5) (2, 32, 33). SOCS1 are induced by cytokines signalling through STAT1, STAT3 and STAT6 and can bind to all JAKs (34), though seemingly favouring STAT1 inhibition, the net result of SOCS1 activation favouring IFN- $\gamma$  suppression (2, 30, 31).

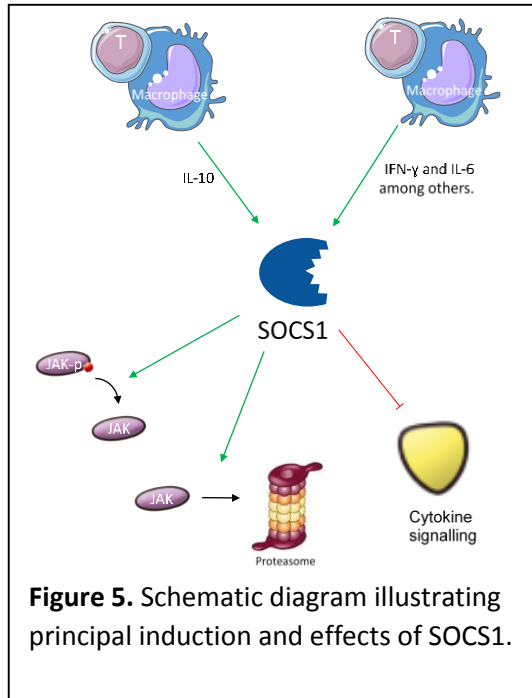
SOCS1 and Tregs have a paradoxical relationship.

Though SOCS1 can inhibit Treg expansion through inhibition of cytokine signalling, such as the IL-2 loop,

it has also been shown that SOCS1 is essential for the regulatory phenotype of FoxP3+ cells.

SOCS1 deficient mice have a larger compartment of FoxP3+ cells, though these lack regulatory cytokine secretion and instead produce IL-17 and IFN- $\gamma$ . FoxP3 downregulates

SOCS1, which might be important to allow for the positive feedback loop of IL-2 secretion, needed for Treg expansion (30, 35, 36).



Both IL-10 and IL-6 induce STAT3 phosphorylation and have the ability to induce SOCS1. In a previous study by our group, using a vector for inflammation dependent locally increased IL-10 production we showed significantly decreased serum levels of IL-6 and an increase in *Socs1* mRNA (20). Dysregulation of the SOCS feedback system can have importance for maintaining manifest autoimmune disease. For instance, Yamana et al

show that under chronic inflammation with prolonged elevated IL-6 levels and high SOCS1 expression, IL-10's ability to effect CD4+ T cells is greatly decreased and substantially higher concentrations of IL-10 is needed to augment STAT3 phosphorylation. This could shown in human RA patients with active disease, both compared to healthy controls and RA patients with inactive disease (33).

## AIM AND SPECIFIC OBJECTIVES

The aim of this study was to generate new hypotheses regarding early mediation of the tolerance underlying tolerance in CIA.

This will be performed by determining the mRNA expression of 96 inflammatory genes and protein expression of selected genes at days 0, 3 and 5 after CIA induction.

## ETHICS

All experiments were approved by the local ethics committee.

## MATERIALS AND METHODS

Male DBA/1 mice that had previously undergone gene therapy with haematopoietic stem cells transduced with LNT-CII/LNTCLIP were used ( $n=20$ ). In brief, mice had been lethally irradiated and transplanted with LNT-CII/LNT-CLIP lentiviral particles (17, 37, 38). Mice were sacrificed before CIA induction (day 0) and three days after (day 3) or five days after (day 5) CIA induction. CIA was induced by subcutaneous injection with rat CII (1 mg/ml) and complete Freund's adjuvant in a total volume of 100  $\mu$ l.

At termination blood, spleen and draining lymph nodes were obtained. Blood samples were collected in Eppendorf tubes with 40  $\mu$ l Heparin LEO 5000 IU/ml (LEO Pharma, Imported by Oripfarm). Plasma was stored at  $-20^{\circ}\text{C}$ . Spleens were collected to previously weighed Falcon tubes containing PBS. Tubes were weighed again to determine the weight of the spleens. A piece of the spleens was taken, embedded in OCT and frozen in isopentane and dry ice for subsequent immunohistochemistry. Another piece of the spleens was frozen immediately in a 2 ml Safe seal tube for PCR preparation. The remaining spleen was prepared as a single cell suspension for FACS analysis. One lymph node was removed to a previously weighed Safe seal tube and frozen on dry ice. The tube and lymph node was weighed again before being prepared for PCR. Several lymph nodes per mouse were prepared as a single cell suspension for FACS analysis. One lymph node per mouse was taken, embedded in OCT and frozen in isopentane and dry ice for immunohistochemistry.

### Quantitative PCR

mRNA was prepared from lymph nodes taken day 0, day 3 and day 5 using RNEasy Mini Kit and QIAcube (QIAGEN) with protocol RNeasy Mini Kit Cells and Tissue with DNase Digest according to the manufacturers instruction, with the exception of using 30  $\mu$ l elution volume

instead of 50  $\mu$ l. RNA concentration was measured using a ND-1000 Spectrophotometer (NanoDrop). cDNA synthesis was made using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) including RNase inhibitor in a Veriti 96 Well Thermal Cycler (Applied Biosystems). cDNA was run in the 384-well microfluid card Taqman Array Mouse Immune Panel (Applied Biosystems/Life Technologies) detecting 90 + 6 genes using a ViiA 7 Real-Time PCR System (Applied Biosystems/Life Technologies).  $\beta$ -actin (*Actb*) and GADPH (*Gadph*) were selected as housekeeping genes. In a previous study, qPCR had been performed using the same Taqman Array on draining lymph nodes from day 14 and day 28. Relative quantification (RQ) was calculated from the sample from a naïve DBA/1 mouse. A separate qPCR was run in a regular 96 well plate for transcription factors *Bcl6*, *Foxp3*, *Gata3*, *Rorc* and *Tbx21* using a ViiA 7 Real-Time PCR System (Applied Biosystems/Life Technologies). For this qPCR  $\beta$ -actin was used as a housekeeping gene. RQ was calculated from the same sample from a naïve DBA/1 mouse

## FACS

Spleen and draining lymph node samples were pressed through a 70  $\mu$ m cell strainer filter. The filter was washed with 10 ml FACS buffer. Cells were pelleted at 300 g for 10 min at 4°C and then resuspended in 0,5 ml FACS buffer. Cells were counted in a NucleoCounter NC-100 (ChemoMetec A/S). Cell concentration was adjusted to  $10^8$  cells/ml. 50  $\mu$ l cell suspension was removed to an Eppendorf tube for cell proliferations. The cells were further stained with Fc-block and the following antibodies CD4 (EF450), CD8 (PE-Cy7), CD25 (FITC), CD44 (APC-H7), CD62L (PerCP) and ICOS (APC) and incubated in dark fridge for 20 minutes before being washed. Cells were then fixed, permeabilized and stained intracellularly for FoxP3 (PE). Fluorochromes minus ones (FMOs) were prepared for FITC, APC-H7, PerCp,

APC and PE. Cells were detected using FACSCanto II (BD sciences) and analysis was made using FlowJo Software (Tree Star Inc.).

### **Cell proliferations**

Cell proliferations were performed using spleens obtained from mice taken at day 5 after CIA induction. Both unstimulated proliferation and CII-proliferation were done. Supernatants were frozen at -20°C. Proliferation was determined as IFN- $\gamma$  production. ELISA for IFN- $\gamma$  was performed using Mouse IFN-gamma DuoSet (R&D Systems, Cat: DY485) according to the manufacturer's instructions. Triplets of supernatant of both stimulated and unstimulated proliferations were used. The plates were analysed using a SpectraMax 340PC384 Absorbance Microplate Reader (Molecular Devices) and SoftMax Pro 5.2 Microplate Data Acquisition & Analysis Software (Molecular Devices).

### **Cytometric bead array**

Blood plasma from days 0, 3 and 5 was used. The plasma had been stored in -20°C. Circulating levels of cytokines were assessed using cytometric bead array (CBA). The Mouse Th1/Th2/Th17 Cytokine Kit (BD, Cat: 560485) was used. The kit detects IL-2, IL-4, IL-6, IL-10, IL-17A, TNF- $\alpha$  and IFN- $\gamma$ . The kit was used following the manufacturer's instructions. Samples were acquired using BD FACSVerse (BD Biosciences). The samples was analysed using FCAP Array v3.0 (Soft Flow, Inc., R&D).

### **Immunohistochemistry**

Immunohistochemical staining with Foxp3, SOCS1 and SOCS3 antibodies was planned. Frozen 6  $\mu$ m sections of one spleen from a previous study was used for optimization of the staining protocol. Sections were fixated with acetone. ImmPress kits from Vector

Laboratories was used for protein blocking. Primary antibody was applied and the sections were left to incubate in a humidity chamber in 2–8°C over night. Endogenous peroxidases were quenched using PBS supplemented with 0.3% H<sub>2</sub>O<sub>2</sub> and 0.1% saponin. Protein block, serum free (Dako Sweden AB, cat: X0909) was used for a second protein block before applying secondary antibody from ImmPress kits. Secondary antibody was allowed to incubate in room temperature for >30 min. ImmPACT AEC Peroxidase (HRP) Substrate (Vector Laboratories Inc, cat: SK-4205) was used as staining reagent. Sections were then stained with Mayer’s hematoxin before mounting with VectaMount AQ Aqueous Mounting Medium (Vector Laboratories Inc, cat: H-5501).

A range of dilutions were tested for each primary antibody. SOCS1 and SOCS3 primary antibodies were tested in the following dilutions 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600. Foxp3 primary antibody was tested in the following dilutions 1:50, 1:100, 1:200 and 1:400. Optimal concentrations are listed in table 1. Negative controls for each secondary antibody were used. During primary antibody incubation these negative control incubated with only antibody dilution buffer (PBS supplemented with 0.1% saponin and 0.5% BSA). See table 1 for specific materials and concentrations.

Protein	Primary antibody	Original concentration	Optimal concentration	Optimal dilution	Secondary antibody and first protein block	Optimal substrate reaction time
Foxp3	Anti-Mouse/Rat Foxp3 Purified clone FJK-16s (eBioscience, cat: 14-5773-82).	0.5 mg/ml	2.5 µg/ml	1:200	ImmPRESS HRP Anti-Rat Ig (Peroxidase) Polymer Detection Kit (Vector Laboratories Inc, cat: MP-7404)	30 min
SOCS1	Rabbit Anti-SOCS1 Polyclonal Antibody (Bioss Antibodies, cat: bs-0113R).	1 mg/ml	1.25 µg/ml	1:800	ImmPRESS HRP Anti-Rabbit Ig (Peroxidase) Polymer Detection Kit (Vector Laboratories Inc, cat: MP-7401)	20 min
SOCS3	Rabbit Anti-SOCS3 Polyclonal Antibody (Bioss Antibodies, cat: bs-0580R)	1 mg/ml	1.25 µg/ml	1:800	ImmPRESS HRP Anti-Rabbit Ig (Peroxidase) Polymer Detection Kit (Vector Laboratories Inc, cat: MP-7401)	10 min

**Table 1.** Detailing materials for immunohistochemistry and optimal concentrations and reaction times for these.



## Statistical analysis

To evaluate the PCR-data and to generate a hypothesis, RQ values from the mRNA qPCR arrays was first investigated in multivariate factor analysis using the software SIMCA (version 13.0.3.0, Umetrics). Principal component analysis (PCA) was used to evaluate the general spread of the data. Further, Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) and Partial least square regression (PLS) was used. OPLS-DA is a discriminant analysis and was used to investigate how gene expression differed between the groups at each time point. Variable importance for the projection (VIP) is a value describing the impact that each variable has on the total variance in the model. At each day, variables with a VIP >1 were noted for their association to either group. The pattern of association over time was then investigated. We limited the investigation to variables with consistent association to either of the groups. Variables with consistent association were further scrutinised; existing literature was consulted for relevancy, the data was checked for outliers and PLS analysis performed. A PLS analysis describes how the variations of one variable correlate with those of the other variables in the selected material. The aim of these statistical analyses was to single out potential candidates for further study.

Univariate analyses were performed using Prism (GraphPad).

## Student's involvement

The mice were already transplanted at the start of the experiment. I observed when Pernilla Jirholt performed plasmid culturing and viral transduction. mRNA isolation, cDNA synthesis and qPCR were performed by Pernilla Jirholt with the me as an observer. I performed the

process on dummy samples. Statistical analysis of qPCR was performed by me. FACS was performed by Berglind Bergmann and FlowJo analysis by me. Cell proliferations was performed by Pernilla Jirholt. ELISA, CBA and immunohistochemistry was performed by me under the supervision of Sofia Andersson.

## RESULTS

### Quantitative PCR

In previous studies we have observed an evident difference in clinical signs and histologic evaluation of arthritis in LNT-CII mice compared to LNT-CLIP mice. To investigate possible explanations to these

	LNT-li-CLIP (n)	LNT-li-CII (n)
Day 0	2	2
Day 3	3	2
Day 5	3	3
Day 14	4	4
Day 28	3	5

**Table 2.** Number of mice that the qPCR array was performed on.

observations in the first days after immunisation we performed the qPCR array at days 0, 3 and 5. Data from mice at day 14 and day 28, gathered in previous studies, was included. Table 2 lists the number of mice with successful mRNA preparation that the qPCR assay could be performed on. The data for day 5 were heavily skewed and riddled with outliers. For that reason day 5 was excluded from the multivariate analyses.

<i>LNT-CII</i>	Hprt1	Socs1	Il12a	Ccl3	Il15	Edn1	Col4a5	Cd28	Ccl2	Ccl19	Tnfrsf1	Tfrc	H2-Ea	Ptgs2	Il7	Cxcr3
Day 0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Day 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Day 14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Day 28	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

<i>LNT-CLIP</i>	Hmox1	Ptprc	Tgfb1	Gzmb	Ece1	Stat3	Csf3	Il17	Selp
Day 0	.	.	.	.	.	.	.	.	.
Day 3	.	.	.	.	.	.	.	.	.
Day 14	.	.	.	.	.	.	.	.	.
Day 28	.	.	.	.	.	.	.	.	.

**Table 3.** List of genes with consistent association to the LNT-CII or LNT-CLIP group. A dot means positive association

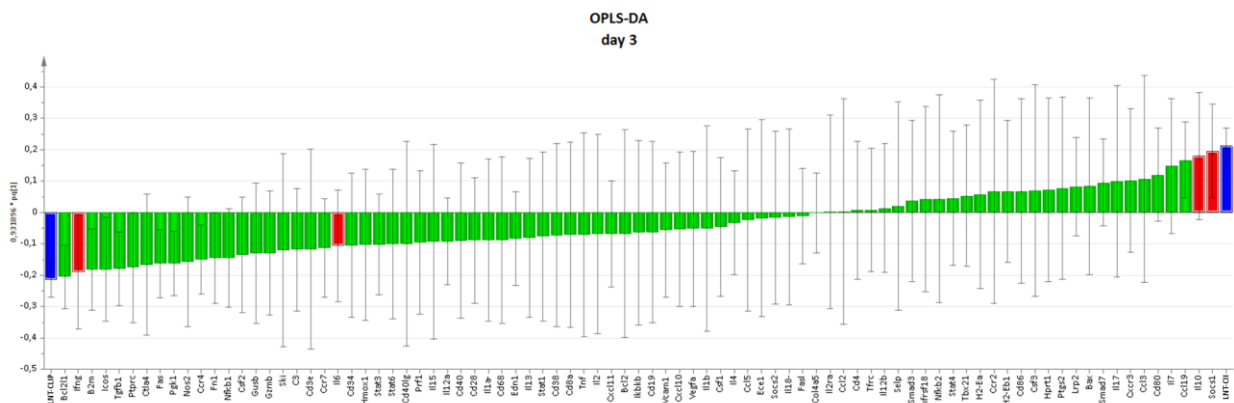
Table 3 lists the genes with consistent association to either group. After reviewing the data and existing literature our three main candidates for further study was *Socs1*, *Hmox1* and *Il12a*. *Hmox1* encodes the protein heme oxygenase 1 (HO-1) which catabolizes heme to biliverdin. Inhibition of HO-1 has been shown to decrease joint inflammation and cartilage destruction as well as inflammatory cytokine levels in CIA (39). *Il12a* encodes a subunit of both IL-12 and recently described anti-inflammatory cytokine IL-35 (40).

We decided to focus on *Socs1*. This decision was based on the limited frames of this report, the amount and quality of existing research and the strength of the findings in our data.

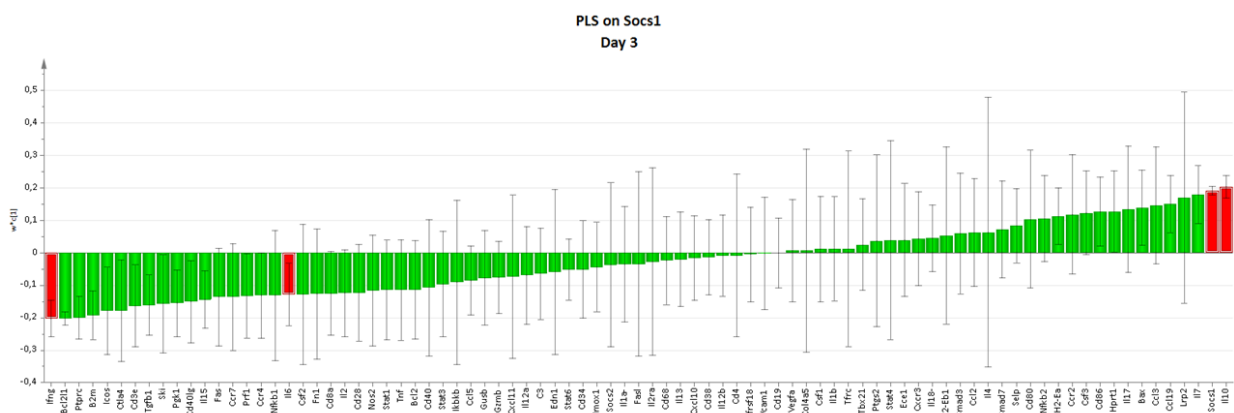
According to the OPLS-DA analysis (i.e. the analysis that sorts the data according to its association to either LNT-CII or LNT-CLIP) at day 3, we found that *Socs1* was the gene strongest associated with the tolerant LNT-CII group along with *Il10* (figure 6A). According to the PLS analysis of *Socs1* (i.e. how parameters co-variate with *Socs1* expression) at day 3, its expression has a positive association with *Il10*, at the same time as it is negatively associated with expression of *Ifng* and *Il6* (figure 6B). In figure 7 the expression of *Ifng*, *Il6*, *Il10* and *Socs1* are plotted over time. In the LNT-CII mice changes in expression of *Il10* and *Socs1* follows the same pattern while *Ifng* follows an inverted pattern. This is particularly marked at day 3 where expression levels of these three genes are the most separated.

No significant differences could be seen in the separate qPCR for transcription factors *Bcl6*, *Foxp3*, *Gata3*, *Rorc* and *Tbx21* (data not shown).

A

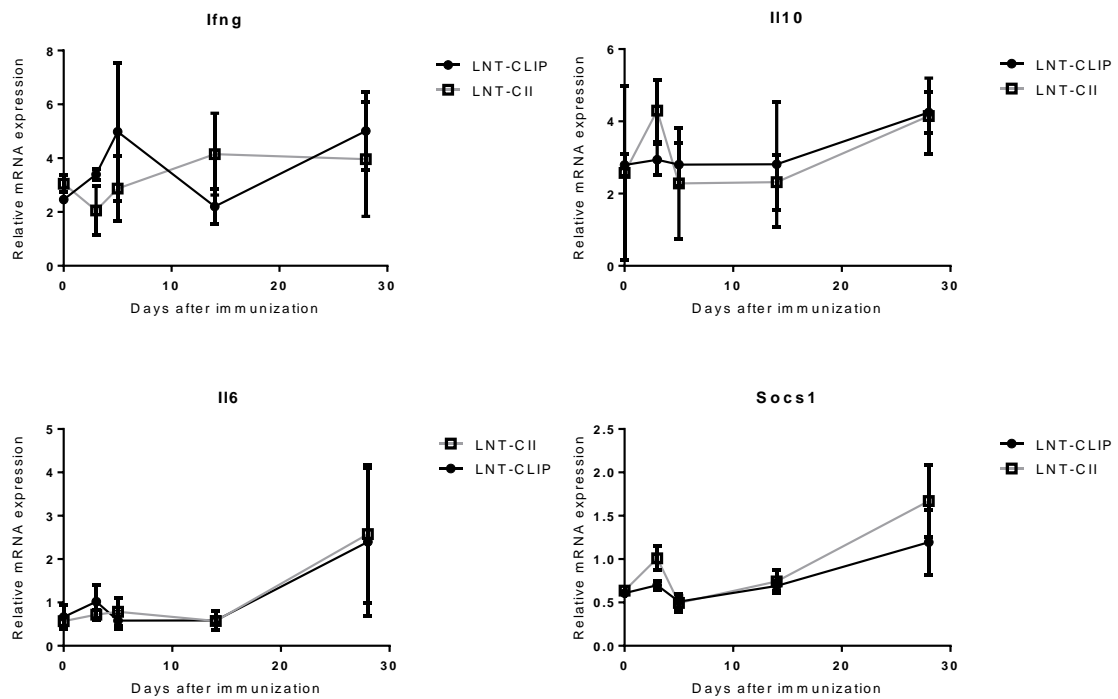


B



**Figure 6. A)** Columns loading plot of OPLS-DA on day 3. This is a discriminant analysis and mice are assigned to groups. A bar above the axis indicates higher expression in the LNT-CII group and a bar below the axis indicates higher expression in the LNT-CLIP group. The bars marked in blue represent the respective groups. Bars for *Socs1*, *Il10*, *Il6* and *Ifng* are marked with red. *Socs1* and *Il10* are the genes with the strongest association to the LNT-CII group. Second to *Bcl2l1*, *Ifng* is the gene strongest associated to the LNT-CLIP group. *Il6* is predominantly associated with LNT-CLIP.

**B)** Columns loading plot of PLS analysis on *Socs1* on day 3. In this analysis mice are not assigned to groups. Both LNT-CLIP and LNT-CII mice taken at day 3 are included. A bar above the axis indicates that expression of the gene covariates in a positive way with *Socs1* and a bar below the axis indicates that expression of the gene covariates in a negative way with *Socs1*. Bars for *Socs1*, *Il10*, *Il6* and *Ifng* are marked with red. The graph illustrates that expression of *Socs1* have a strong positive association to the expression of *Il10* and a strong negative association to the expression of *Ifng* and *Il6*.

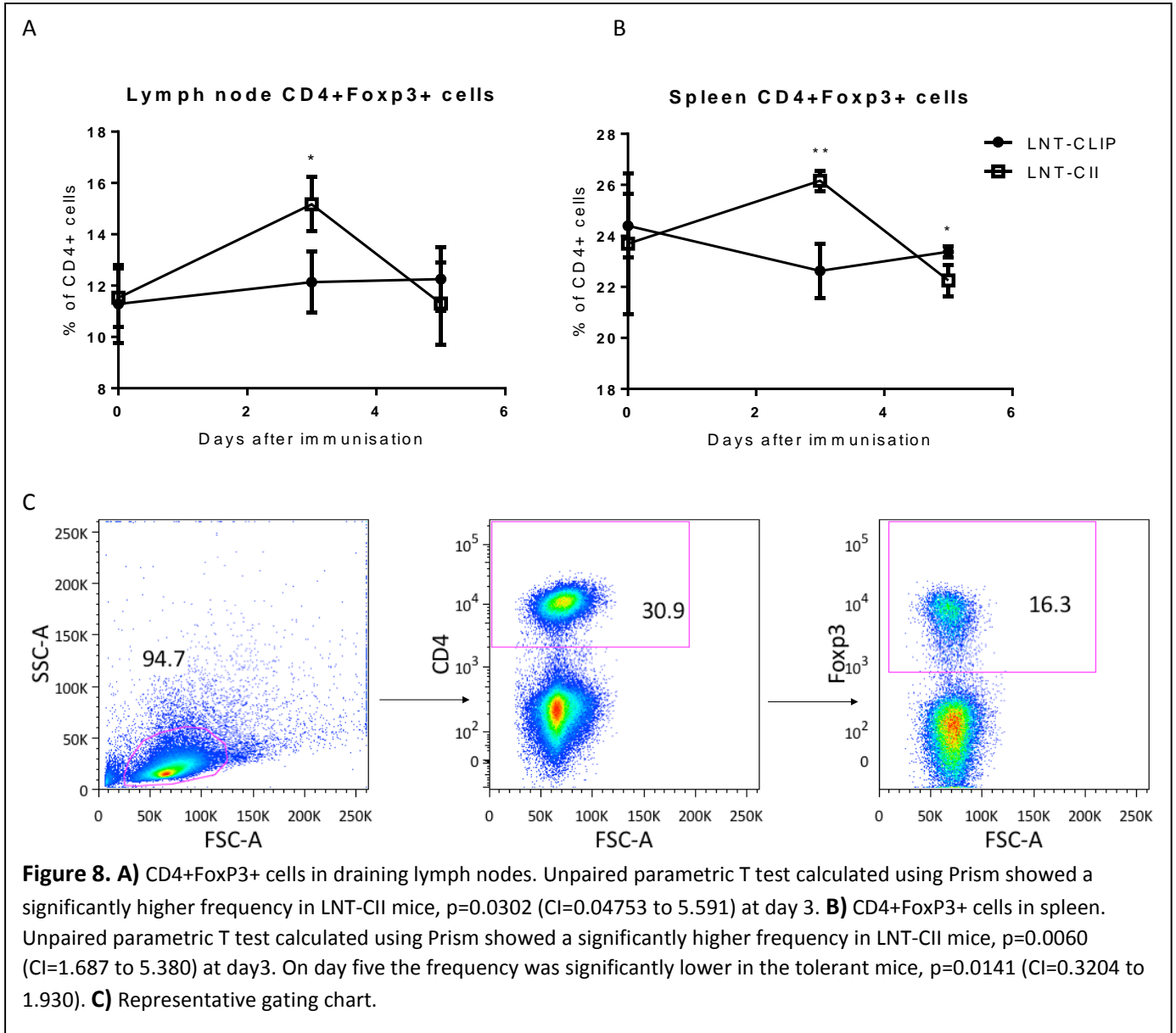


**Figure 7.** *Ifng*, *Il6*, *Il10* and *Socs1* plotted over time. At day 3 the most evident separation between the groups can be observed. In the LNT-CII mice both *Il10* and *Socs1* are upregulated at day 3, while *Ifng* is downregulated. Due to mass significance statistical calculations cannot be performed. It can be noted that over time, the levels of *Il10* expression and *Socs1* expression in the LNT-CII mice seems to share the same pattern, while expression of *Ifng* exhibits the inverted pattern.

## FACS

To investigate T cell subsets FACS was performed on spleen and draining lymph nodes at day 0, day 3 and day 5. The frequency of regulatory T cells, defined as CD4+FoxP3+ cells, is significantly increased at day 3 in both draining lymph nodes and spleen of LNT-CII mice (figure 8A and B).

At day 5 the proportion of CD4+FoxP3+ cells were normalised in draining lymph nodes. In the spleen there was a significantly decreased frequency of CD4+Foxp3+ cells in LNT-CII mice. This suggests that events crucial to tolerance takes place very early after induction of arthritis.





## ELISA

The results from the ELISA was inconclusive as all samples was negative (data not shown).

We suspect that proliferations failed and the cells were dead. The standard curve obtained had a  $R^2$  value of 1.

## Cytometric bead array

Results were inconclusive. Results for TNF were not available as the standard curve for had a  $R^2$  value <98% why the software wouldn't fit them. All other standard curves had a  $R^2$  value >98%. Results for IL-2, IL-4, IL-10 and IFN- $\gamma$  were all, or to an extent not allowing comparison between groups, below standard range. Concentrations detected of IL-17A and IL-6 contained no significant difference between the groups. Results shown in table 4.

Results File	IL-10	Message	IL-17A	Message	TNF	Message	IFN- $\gamma$	Message	IL-6	Message	IL-4	Message	IL-2	Message
R1	0,00	II	1,92		N/A		0,65		1,79		0,00	II	2,58	I
R2	0,00	II	1,36		N/A		0,00	II	0,46		0,00	II	0,00	II
R3	0,00	II	1,12		N/A		0,00	II	3,43		0,00	I	2,00	I
R4	0,00	II	2,05		N/A		0,00	II	2,41		0,00	II	1,41	I
R5	0,00	II	1,50		N/A		0,47		2,47		0,00	II	3,37	
R6	0,00	II	0,24		N/A		0,00	II	4,74		0,00	II	0,00	II
R7	0,00	II	1,50		N/A		0,47		4,08		0,00	II	1,12	I
R8	0,00	II	1,12		N/A		0,96		0,04		0,00	II	1,83	I
R9	0,00	II	1,50		N/A		1,03		3,49		0,00	I	0,00	II
R10	0,00	II	0,70		N/A		0,47		2,47		0,00	II	1,41	I
Q1	0,00	II	1,96		N/A		0,09	I	0,00		0,00	I	0,00	II
Q2	0,00	II	3,18		N/A		0,32		2,60		0,00	I	1,12	I
Q3	0,00	II	1,17		N/A		0,00	II	0,00	I	0,00	II	0,00	II
Q4	0,00	II	1,36		N/A		0,37		3,43		0,00	II	2,16	I
Q5	0,00	II	1,12		N/A		0,19	I	10,47		0,00	II	0,00	II
Q6	0,00	II	0,87		N/A		0,00	II	0,52		0,00	II	0,00	II
Q7	0,00	II	0,59		N/A		0,77		2,10		0,00	I	0,00	II
Q8	0,00	II	1,69		N/A		0,77		4,81		0,00	II	1,12	I
Q9	0,00	II	1,07		N/A		0,00	II	0,70		0,00	II	1,41	I
Q10	0,00	II	1,45		N/A		0,00	II	0,10		0,00	II	1,83	I

**Table 4.**

Results from CBA.

LNT-CLIP: R1-3 = d0, R4-6 = d3, R7-10 = d5

LNT-CII: Q1-3 = d0, Q4-6 = d3, Q7-10 = d5

Concentrations in pg/ml.

Message: I: Fitting; Below standard range II: Below standard range and out of invertable range

## DISCUSSION

This report creates new hypotheses with respect to the underlying immunological mechanisms in antigen-specific tolerance in CIA. We show that tolerant LNT-CII mice up-regulates the *Socs1* gene at day 3 after CIA induction. Similarly, the *Il10* gene is up-regulated while *Infγ* is down-regulated at the same point. The PLS analysis on all mice taken day 3 suggests that expression of *Socs1* have a positive correlation expression of *Il10* and negative association with genes for pro-inflammatory cytokines IFN- $\gamma$  and IL-6. Due to mass significance and small number of observations statistical tests cannot be performed, but we would argue that this pattern strengthens the case against this being merely coincidental. Further support can be found in the seemingly interdependent pattern of *Socs1*, *Il10* and *Infγ* when plotted over time (figure 8). We have found a significant increase in CD4<sup>+</sup>FoxP3<sup>+</sup> T regulatory cells in tolerant LNT-CII mice at day 3, which could explain an increased *Il10* expression. The CD4<sup>+</sup>CD44<sup>+</sup>Foxp3<sup>+</sup> population is known for high IL-10 secretion. There was a tendency towards simultaneous increase in this population in the LNT-CII mice, though none significant (data not shown). IL-10 is known to be able to induce *Socs1* and in our group's previous study (20) with local inflammation dependent overexpression of IL-10 we found an increased *Socs1* expression in draining lymph nodes and reduced IL-6 serum levels, which supports the findings of this study. There are many reports supporting a protective role of IFN- $\gamma$  in established RA. However, this does not necessarily contradict our findings as they generally describe established disease. Studies of early RA are scarce due to the intrinsic delay of diagnosis from onset of disease. IFN- $\gamma$  has been reported to mediate pharmacological inhibition of CIA (26, 41). Further study is needed to fully evaluate the impact of the temporary early suppression of IFN- $\gamma$  seen in this study.

Our hypothesis is that the process leading to the tolerance is initiated even before immunisation. We propose that immediate differences in the inflammatory response to immunisation diverts the LNT-CII animals down a tolerant path. We believe that a part of this process is illustrated by our observations at day 3: Expansion of the Treg compartment with increased IL-10 expression in the LNT-CII mice induces increased expression of SOCS1. SOCS1 in turn exerts a general negative feedback on the JAK/STAT pathway, decreasing signalling by and mRNA expression of pro-inflammatory cytokines such as INF- $\gamma$  and IL-6. How big a part this is of the whole picture cannot be concluded within the frame of this study.

#### Protein expression of IL-16, IL-10, IFN- $\gamma$ and SOCS1

To verify the mRNA expression of chosen genes on a protein level, several methods were used. To detect IFN- $\gamma$ , we performed an ELISA on supernatants from the proliferation experiment. However, no detectable levels of cytokines were found. This could be due to multiple reasons: 1) The proliferations failed and the cells were dead, 2) our detection threshold was too high and that a lower standard curve might have been more suitable.

To detect various cytokines in plasma we performed a CBA analysis. Unfortunately we could not detect any cytokines. Some possibilities as to why might be: 1) Many of the cytokines measured, such as IL-2 and IL-10, is primarily believed to have paracrine functions and does not likely reach detectable concentrations in serum even during high secretion, 2) the plasma had been frozen which may have influenced the protein configuration and thus antibody binding sites, 3) the CBA might not be sensitive

To detect SOCS1, 3 and Foxp3 in draining lymph nodes by immunohistochemistry, we titred all adequate antibodies. However, due to lack of time this part of the project was not completed, but will be carried out in the future.

### Strengths and weaknesses

The main weakness of this work is the small number of observations and mass significance in a 96 gene array. The low quality of data from qPCR performed from day 5 lead to exclusion of this time point, which was a loss. The failure to verify mRNA data on a protein level is an apparent weakness.

### Future applications

Re-establishment of tolerance in autoimmune diseases would be the ultimate treatment. To achieve that, we need to understand the underlying immunological mechanisms in greater detail. Gene therapy can be on option for inborn diseases such as severe immunodeficiencies but presently not for autoimmune conditions. However, research like ours leads to a deeper understanding of the disease pathogenesis. This improves the characterisation of the individual patient's pathogenesis and help the physician to personalise the pharmacologic treatment. Research like ours also reveal new immunological events that can provide novel treatment targets. An example closely related to this report is the recent introduction of JAK inhibitors the treatment of RA.

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## POPULÄRVETENSKAPLIG SAMMANFATTNING

Med immunologisk tolerans menar man immunsystemets förmåga att tåla sig den egna kroppen, men samtidigt reagera kraftfullt på liknande komponenter från främmande organismer. Trots en mängd säkerhetssystem, kan denna tolerans brytas och en immunitet mot kroppens egna beståndsdelar utvecklas. Det är denna förmåga till immunitet som utnyttjas när vi vaccinerar oss. Immunitet mot sig själv benämns som autoimmunitet.

Ledgångsreumatism kallas medicinskt reumatoid artrit (RA). Artrit betyder just ledinflammation. RA är en vanlig autoimmun sjukdom som drabbar upp till 1% av västerländska befolkningar. Obehandlad kan sjukdomen leda till mycket handikappande ledförstörelse. Man kan inte bota den här typen av sjukdomar, utan behandling av RA inriktar sig på att dämpa immunsystemet. Trots att vår förmåga att hindra sjukdomens förlopp har ökad dramatiskt de senaste decennierna medför sådan behandling alltid en nedtryckning av immunsystemets gynnsamma funktioner. Man blir alltså känsligare för och riskerar allvarliga infektioner. Den ultimata behandlingen vore att återskapa toleransen och därmed bota sjukdomen.

Vi använder en sjukdom hos möss som liknar RA, en så kallad musmodell för RA, för att studera sjukdomens mekanismer. Den musmodell vi använder kallas collageninducerad artrit (CIA) och bygger på att möss immuniseras mot komponenter i ledbrösket. De får då en sjukdom som liknar RA.

Vi har utvecklat ett system för att med hjälp av skraddarsydda virus introducera en ny gen i mössens immunceller. De som får denna gen utvecklar inte sjukdomen trots att de immuniseras på samma sätt. Vi använder den här modellen för att studera mekanismerna bakom tolerans i hopp om att hitta nya sätt att behandla RA.

Nu har vi för första gången studerat de första dagarna efter immunisering för att försöka hitta tidiga mekanismer som förhindrar sjukdomsutveckling. Vi har tittat på vilka typer av immunceller som finns samt hur mycket aktivitet de har i ca 90 gener relaterade till inflammation.

Vi såg att en inflammationsdämpande typ av cell kallad regulatorisk T-cell var vanligare hos de toleranta mössen tre dagar efter immunisering. Vid samma tidpunkt var gener för antiinflammatoriska ämnen uppreglerade i dessa möss samtidigt som gener för proinflammatoriska ämnen var nedreglerade. Vi har också identifierat ett protein kallat SOCS1 som vi tror kan spela en viktig roll i att skapa den här skillnaden.

Virusbaserade metoder såsom denna är ännu inte lämplig att använda på människor på bred front. Dessutom ger vår metod störst effekt om den utförs före sjukdomsutveckling. Däremot kan forskning, liksom vår, leda till ökad förståelse för vilka som får sjukdomen och varför. Den kan också leda till att vi bättre förstår vilken patient som kommer att svara på vilket utav våra många nya läkemedel. En mer skraddarsydd behandling skulle kunna leda till bättre resultat med mindre biverkningar.

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