Disease-Dependent Local IL-10 Production Ameliorates Collagen Induced Arthritis in Mice

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Abstract
Rheumatoid arthritis (RA) is a chronic destructive autoimmune disease characterised by periods of flare and remission. Today’s treatment is based on continuous immunosuppression irrespective of the patient’s inflammatory status. When the disease is in remission the therapy is withdrawn but withdrawal attempts often result in inflammatory flares, and re-start of the therapy is commenced when the inflammation again is prominent which leads both to suffering and increased risk of tissue destruction. An attractive alternative treatment would provide a disease-regulated therapy that offers increased anti-inflammatory effect during flares and is inactive during periods of remission. To explore this concept we expressed the immunoregulatory cytokine interleukin (IL)-10 gene under the control of an inflammation dependent promoter in a mouse model of RA - collagen type II (CII) induced arthritis (CIA). Haematopoetic stem cells (HSCs) were transduced with lentiviral particles encoding the IL-10 gene (LNT-IL-10), or a green fluorescence protein (GFP) as control gene (LNT-GFP), driven by the inflammation-dependent IL-1/IL-6 promoter. Twelve weeks after transplantation of transduced HSCs into DBA/1 mice, CIA was induced. We found that LNT-IL-10 mice developed a reduced severity of arthritis compared to controls. The LNT-IL-10 mice exhibited both increased mRNA expression levels of IL-10 as well as increased amount of IL-10 produced by B cells and non-B APCs locally in the lymph nodes compared to controls. These findings were accompanied by increased mRNA expression of the IL-10 induced suppressor of cytokine signalling 1 (SOCS1) in lymph nodes and a decrease in the serum protein levels of IL-6. We also found a decrease in both frequency and number of B cells and serum levels of anti-CII antibodies. Thus, inflammation-dependent IL-10 therapy suppresses experimental autoimmune arthritis and is a promising candidate in the development of novel treatments for RA.


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Introduction
Rheumatoid arthritis (RA) is a systemic chronic autoimmune disease that mainly affects the joints and ultimately leads to severe bone and cartilage destruction. The clinical course of the disease is discontinuous and characterised by spontaneous remissions and exacerbations. The aetiology in RA is largely unknown but for some reason the immune system - which normally protects us from exogenous pathogens - is dysregulated and has lost its normal tolerance to endogenous (self-) structures and mediates an inflammatory attack against e.g. the joints. Today’s treatment is based on continuous immunosuppression either by conventional disease modifying anti-rheumatic drugs such as methotrexate and/or by biological agents targeting specific proteins e.g. TNF. Unfortunately these treatment modalities can cause side effects such as severe infections and, in addition, attempts to withdraw therapies in established RA often leads to flares [1]. To overcome these hurdles, disease-regulated therapy appears ideally suited, as it would allow intrinsic expression of the immunosuppressive therapy only during inflammatory conditions i.e. during disease flares but not during periods of remissions. This approach has been used successfully in experimental autoimmune encephalomyelitis (EAE) where, by means of transcriptionally targeted gene therapy, a T cell targeted IL-2 promoter controlling IL-10 production delayed onset and progression of EAE [2]. It has also been shown that disease-regulated IL-4 expression achieved via the IL-1/IL-6 promoter can protect against cartilage destruction in CIA [3].

Interleukin-10 is produced by a multitude of cell types during an immune response, where one of its main functions is to limit the ongoing response in order to protect the host from excessive immune mediated tissue destruction [reviewed in [4]], which is one of the characteristics in RA. Support for a role of IL-10 in RA comes from mouse models: in the CIA model, treatment with anti-
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Figure 1. Lentiviral gene constructs and clinical development of arthritis. (A) Lentiviral constructs: LNT-GFP and LNT-IL-10. LTR: long terminal repeat, cPPT: central polypurine tract, pA: polyadenylic acid tail, WPRE: Woodchuck post-transcriptional regulatory element, IL-1E: Interleukin-1 enhancer, IL-6 promoter. (B) Severity of arthritis (mean arthritis score ± SEM). LNT-GFP (day 0–42 n = 18, day 44–49 n = 10) and LNT-IL-10 (day 0–42 n = 25, day 44–49 n = 14)). (C) Histopathological severity of synovitis and cartilage and bone erosivity measured as histological severity score (Y-axis) ranging from 0–3. Data in figure 1B and C were analysed by Mann-Whitney U-test. Closed circles represents LNT-GFP and open circles LNT-IL-10 mice. Bars in 1C represent the median. doi:10.1371/journal.pone.0049731.g001
Discussion

Our report shows that increased local, but not systemic, levels of IL-10 conferred by disease-driven gene therapy delays the progression of CIA in mice. A precise and restricted increase in IL-10, produced by B cells and other APCs, ameliorates the course and severity of arthritis. Based on our data, a possible scenario would be that the increase in IL-10 upregulates SOCS1 resulting in a decrease in serum levels of IL-6. This in turn results in a decrease in both frequency and number of B cells and anti-CII antibody levels, accompanied by reduced severity of arthritis.

IL-10 is a potent pleiotropic cytokine that is produced e.g. by monocytes, macrophages, T and B cells. This cytokine has the capacity to inhibit synthesis of pro-inflammatory cytokines such as IL-2, IFN-γ, TNF-α and importantly IL-6 [4]. It has earlier been shown that systemically increased IL-10 levels suppresses the frequency and severity of CIA [17,18,19,20,21]. The inflammation-dependent IL-1/IL-6 promoter has low basal activity, which significantly increases during acute inflammatory conditions [13]. We found that this promoter, driving the IL-10 gene expression, does not induce increased systemic (serum) levels of IL-10 during the course of arthritis in vivo, but a locally increased IL-10 expression in lymph nodes; particularly in B cells and other APCs. Whether the B cells in the LNT-IL-10 mice are IL-10-producing regulatory B cells [17] is currently unknown, although it is possible as such cells have been found to reduce the severity of arthritis [18,22]. Our data are supported by those of others [12], where it was recently found that a local and inflammation-dependent increase in IL-10 produced by endothelial cells results in suppressed development of zymosan induced arthritis in mice.

Interleukin-6 has been found to contribute to the development of synovitis as well as cartilage and bone destruction in autoimmune arthritis [reviewed in [23]]. As expected, IL-6 was almost absent in the LNT-IL-10 mice but not in the arthritic control group. IL-6 is regulated by a multitude of mechanisms including SOCS1 and 3 e.g. SOCS1 down regulates its expression [24]. The SOCS adaptor proteins are in turn induced by IL-10 [15]. In fact, increased expression of both SOCS1 and 3 have been shown to decrease the severity of arthritis [15,25,26]. In our system, increased production of IL-10 in lymph nodes coincides with elevated mRNA levels of SOCS1 and decreased levels of IL-6. It is also known that IL-6 in the presence of TGF-β drives ROR-γt expression in naive T cells to Th17 cells, while the absence of IL-6 induces FoxP3 expression and expansion of T

Figure 2. Levels of IL-10 mRNA, intracellular IL-10 production and SOCS expression (A). Levels of IL-10 mRNA expression in lymph nodes at day 42 in LNT-GFP or LNT-IL-10 mice. (B) The amount of IL-10/cell measured as geometric mean fluorescent intensity (MFI) in lymph node CD19+MHC II+B cells, (C) in lymph node CD19+MHC II+non-B APCs (D) in splenic B cells, (E) in splenic non-B APCs. (F) Typical gating for intracellular cytokine staining showing one sample from an LNT-GFP mouse and an LNT-IL-10 mouse (G) Levels of mRNA SOCS1 and 3 expression in draining lymph nodes at day 42. In figure 2A–E and G data were analysed by Mann-Whitney U-test. Closed circles represents LNT-GFP and open circles LNT-IL-10 mice.

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regulatory cells [27,28,29]. At the studied time points, no
differences in the number of T regulatory cells or serum levels of
IL-17 could be detected, suggesting that this mechanism is less
likely.

The frequency of B cells is decreased both locally in lymph
nodes and systemically in spleen of LNT-IL-10 mice compared
with controls. This effect might be attributed mainly to decreased
IL-6 levels as the cytokine originally was identified as a B-cell
differentiation factor and plays an important role in the de-
velopment of antibody-producing plasma cells [30]. Beside the fact
that fewer B cells can lead to lower levels of anti-CII IgG
antibodies (which also could be due to a less inflammatory status),
the beneficial effects of a reduced B cell population is well
described in the outcome of human RA by the use of B cell
depleting anti-CD20 antibodies [31].

Our study suggests that inflammation-dependent IL-10 pro-
duction causing locally increased levels of IL-10, increased SOCS1
mRNA and a decrease in systemic IL-6 levels ameliorate the
outcome of CIA in mice. However, the concept needs to be tested
in human RA, as the role of IL-10 in RA patients is far from
clarified: RA patients have significantly elevated levels of IL-10 in
synovial fluid [32] while the expression of IL-10 receptors are
reduced in synovial tissue [33] compared with osteoarthritic
controls, and treatment with systemic recombinant IL-10 in
human RA patients has so far not shown any convincing results
[34]. Although these findings appear disappointing they do not
contradict our data. Rather, they suggest that the anti-arthritis-
egenic effect might be dependent on a requirement for localised
rather than systemic IL-10 treatment.

Taken together, our study demonstrates that disease-regulat-
ed therapy mediated by IL-10 has a beneficial outcome on arthritis
development in CIA and provides a step towards disease-regulated
therapies in human autoimmune arthritis.

Materials and Methods

Cloning of Inflammation Dependent GFP and IL-10
Lentiviral Vectors

To generate the inflammation dependent lentiviral vector
encoding the green fluorescent protein (GFP), pGL3B containing
the hybrid promoter (IL-1/IL-6) was digested with BglII/Hind1I. The
1.3 kilo base pair (kbp) fragment was subcloned into
a modified pBluescript vector, to have additional cloning sites,
Pac1/Asc1. By using Pac1/Asc1 the promoter fragment was further
subcloned into pHR’SIN-cPPT-SEW containing both GFP and
woodchuck post-transcriptional regulatory element (WPRE). The
vector was named LNT-GFP. To create the inflammation
dependent lentiviral encoding IL-10, the eGFP gene was digested
from the LNT-GFP by Pme1/Sal1. A pCI vector containing the IL-
10 cDNA was digested with Not1/Xho1 and replaced the eGFP
gene, creating the LNT-IL-10. All restriction enzymes and ligases
were obtained from New England Biolabs (NEB Ipswich, MA,
USA).

Production of Lentiviral Particles

Vesicular stomatitis virus-G (VSV-G) pseudotyped lentivirus
was produced by transient transfection of 293FT cells with three
plasmids: one of the self inactivating transfer vector plasmids
(LNT-GFP and LNT-IL-10); the multi-deleted packaging plasmid
pCMV D8.74; and the VSV-G envelope pMD.G2 using calcium
phosphate co-precipitation. At 72 h post transfection, the medium
was harvested and concentrated by ultracentrifugation at
90,000 g. The pellets were resuspended in PBS containing 2%
FCS and stored at −80°C.

Lentiviral Particle Titration

Viral titer was determined on NIH/3T3 (American Type
Culture Collection, Manassas, VA, USA) mouse fibroblast cell line
using real time-PCR directed towards the WPRE sequence.
Vector copy numbers are normalised to titin gene copies. WPRE
forward primer: 5′ GGC ACT GAC AAT TCC GTG GT 3′,
WPRE reverse primer: 5′ AGG GAC GTA GCA GAA GGA CG
3′ and WPRE probe 5′ 6-FAM- ACG TCC TTT CCA TGG
CTG CTC GC- TAMRA- 3′. Titin forward primer: 5′ AAA
ACG AGC AGT GAC GTG AGC 3′, titin reverse: 5′ TTT AGT
CAT GCT GCT AGC GC 3′ and titin probe: 5′ 6-FAM- TGG
ACG GAA GCC TCT CTT CCA TGG CTC GC- TAMRA- 3′. All
primers were obtained from Sigma-Aldrich AB (St Louis, MO,
USA) and probes from Applied Biosystems and the assay was run

Figure 3. Levels of IL-6 and anti-CII antibodies

(A) Serum protein levels of IL-6 (B) and serum levels of anti-CII IgG were analysed at days
29 and 42 after CII immunisation. Analysed by Mann-Whitney U-test.
Closed circles represents LNT-GFP and open circles LNT-IL-10 mice.
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with Taqman Universal PCR Mastermix (Applied Biosystems, California, USA) on 7500 Real Time PCR System (Applied Biosystems).

Mice

Male DBA/1 mice were obtained from Taconic (Europe A/S, Ry, Denmark) and housed in a pathogen-free barrier facility (12-hr light/12-hr dark cycle) and fed rodent chow. All animal studies were approved by the local Animal Ethics Committee.

Inflammation-dependent Production of IL-10 in vitro

To verify inflammation-dependent IL-10 production, bone marrow was harvested from femur and the hip bone from DBA/1 mice and HSCs were isolated with negative selection using EasySep Mouse Hematopoietic Progenitor Cell Enrichment Kit (Stemcell Technologies, Manchester, UK). After isolation, HSCs were resuspended in StemSpan with 1% penicillin/streptomycin and the following cytokines (100 ng/ml mSCF, 100 ng/ml Flt-3L, 100 ng/ml IL-11, 20 ng/ml IL-3) and cultured in 12 well plates at a concentration of 1 × 10^6 cells/ml. The cells were transduced with lentiviral constructs LNT-GFP and LNT-IL-10 at MOI ranging from 0 to 80. The next day the media was changed to a media promoting differentiation of haematopoetic cells to bone marrow derived macrophages containing DMEM supplemented with 10% FCS, 10% L929- conditioned media, 20 mM HEPES and 50 μM 2-mercaptoethanol. After 9 days of differentiation the cells were stimulated with 100 ng/ml LipoPolySaccharide (LPS) or media for 24 h. Supernatants were collected and analysed by mouse Duoset IL-10 ELISA (R&D Systems, Abingdon, UK) according to the manufacturers instructions.

Bone Marrow Transplantation

To minimize risk for infections during transplantation, both donor and recipient mice were treated with the antibiotic (enrofloxacin) Baytril one week prior to and two weeks after the transplantation. Haematopoetic stem cells were harvested, isolated from donor mice as described in the paragraph above and further transduced with lentiviral constructs LNT-GFP and LNT-IL-10 at MOI 75 and incubated at 37°C overnight. The next morning, cells were washed with PBS twice, counted and resuspended at a concentration of 2.4 × 10^5 cells/200 μl. The recipient mice were irradiated with 8.5 Gy and intravenously reconstituted with transduced HSCs (2.4 × 10^5). Mice were repopulated for 12 weeks before induction of arthritis.

Assessment of in vivo Transgene Integration by PCR

To detect vector integration in bone marrow, spleen and synovium 18 weeks after transplantation of transduced HSCs, DNA was prepared using the QIAmpli DNA mini kit (Qiagen, Solna, Sweden) according to the manufacturer’s instructions and the WPRE was amplified with primers and probes described above.

Collagen Type II Induced Arthritis

Two independent experiments were performed and the data were pooled. Arthritis was induced 12 weeks after bone marrow transplantation by a subcutaneous (sc) injection of chicken CII (Sigma-Aldrich AB) (1 mg/ml) in complete freund’s adjuvant (Sigma-Aldrich AB) in a total volume of 100 μl. The mice were boosted sc with CII (1 mg/ml, 100 μg/mouse) in incomplete
fluen's adjuvant (Sigma-Aldrich AB) at day 21 after CII immunisation. All mice were followed individually and checked daily. Clinical arthritis and severity was assessed by an evaluator blinded to the treatment groups. Finger/toe and ankle/wrist joints were inspected and arthritis was defined as visible erythema and/or swelling. To evaluate the severity of arthritis, a clinical scoring (arthritic index) was carried out using a system where macroscopic inspection yielded a score of 0–3 points for each limb. We define our scoring system as follows: 0—no arthritis, 1—mild arthritis (mild swelling and a subtle erythema of the evaluated joint), 2—moderate arthritis (moderate swelling and a more pronounced erythema compared to score 1), 3—severe arthritis (profound swelling and erythema). The total score per animal and time point is calculated by adding up the scores from all four paws. The mice were bled at day 29. At day 42 blood, joints, spleen and lymph nodes were obtained. Histopathologic examination of the joints was performed after routine fixation, decalcification, and paraffin embedding. Tissue sections from fore and hind paws were cut and stained with hematoxylin–eosin. All the slides were coded and evaluated by two blinded observers. The specimens were evaluated with regard to synovial hypertrophy, pannus formation, and cartilage/subchondral bone destruction. The degree of synovitis and destruction in every joint concerning finger/toes, wrists/ankles, elbows, and knees was assigned a score from 0 to 3. Occasionally one paw was missing in the histological sections, or embedded in such a way that it was impossible to evaluate the degree of synovitis and bone/cartilage destruction. Therefore, the total score per mouse was divided by the number of joints evaluated.

**Determination of mRNA Levels of IL-10 in Lymph Nodes**

RNA was isolated from lymph nodes using RNeasy mini kit (Qiagen). The RNA quality was analysed using a Experion Bioanalyzer on a Experion RNA StdSens chip (Bio-Rad laboratories Inc., USA) prior to cDNA synthesis with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The expression of IL-10 gene was analysed using primers IL10 FW 5′-CATTGTAATTTCCGAGTGAGGA and RV 5′-TGCCTCCCTGCTTCTT. The gene expression was normalised to β-actin analysed with primers FW 5′-CTGACAGGATTAGGAAAGGAC-3′ and RV 5′-GCCACCGATTCACAGAGT. The reactions were amplified using Power SYBR green PCR Master Mix (Applied Biosystems) and analysed on a Viia7 system (Applied Biosystems).

**Fluorescence Activated Cell Sorting (FACS) Analyses and Cell Counting**

To detect intracellular cytokine expression in different cell populations, a single cell preparation of lymph node and spleen was performed. The total number of cells in spleen was counted (Nucleocounter, ChemoMetec AS, Denmark). For FACS stainings, 1×10⁶ lymph node or spleen were placed in 96-well plates and pelleted (3 min, 300 g, 4°C). To avoid nonspecific binding via Fc-receptor interactions, cells were incubated with Fc-block (2.4G2, BD Biosciences, San Jose, CA, USA) for 10 min at room temperature. Antibodies used were anti-CD19 (clone ID3), anti-CD4 (clone RM4-5), anti-IL-10 (clone JES5-16E3) and anti-IFN-γ (clone XM12.1) purchased from BD Biosciences, anti-I-A/ I-E (clone M5/114.15.2 purchased from BioLegend, San Diego, CA, USA) and FoxP3 (clone FJK-16s), and IL-17 (clone eBio1B7), purchased from eBioscience (San José, CA, USA). All surface marker antibodies were diluted in FACS-buffer (PBS containing, 1% FCS and 0.5 mM EDTA). For intracellular staining with anti-IL-10 or isotype controls the cells were permeabilised using the FoxP3/Transcription Factor Staining Buffer set from eBioscience and antibodies diluted in 1×PERM buffer included in the kit. The antibodies were directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), V450 and APC-H7. Cells were stained as previously described and gating of cells was performed using fluorochrome minus one settings [35] and detected by FACS-Canto II™ (BD Biosciences). Gating strategy for expression of IL-10 was included in Supplemental Figure 2. Analysis with respect to the number of cells and mean fluorescence intensity (geometric mean) were performed using FlowJo Software, Tree Star Inc. (Ashland, OR, USA).

**Determination of SOCS Expression in Lymph Nodes**

RNA was isolated from lymph nodes using RNAeasy mini kit (Qiagen). The RNA quality was analysed using a Experion Bioanalyzer on a Experion RNA StdSens chip (Bio-Rad) prior to cDNA synthesis with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The gene expression of SOCS3 was analysed using primers FW 5′-CTGGTACTGAGCGACCTCTCT-3′ and RV 5′-CCGTGACAGTCTTTCCCGAGAA-3′, the expression of SOCS1 was analysed using primers SOCS1 FW 5′-AAGGAATCTAGTGATCAGCGGAT-3′ and RV 5′-CCGTGGCTGGCAGAGAAC-3′. The gene expression was normalised to β-actin analysed with primers FW 5′- CTGACAGGATGCAGAGAAGGAGATTACT and RV 5′- GCCACCGATTCACAGAGT. All reactions were amplified using Power SYBR green PCR Master Mix (Applied Biosystems) and analysed on a Viia7 system (Applied Biosystems).

**Determination of Cytochrome Production**

Blood was centrifuged at 7000 g for 10 min. Serum was collected and stored at −20°C for further analysis. Previously prepared spleen cell culture were stimulated for 72 hours with denatured chicken CII 50 μg/ml, supernatants collected and kept in −20°C until further analysis. Two independent experiments were performed. In the first experiment serum protein levels of IL-10 were measured in serum by Duoset ELISA (R&D systems) according to the manufacturer’s recommendations and detected on Spectra Max 340PC (Molecular Devices). In the second experiment serum levels of Th1, Th2, Th17 and Th22 specific cytokines (IL-12, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-21, IL-22, IL-27 and IFN-γ) were measured using FlowCytomix Multiple Analyte Detection Mouse Th1/Th2/Th17/Th22 13plex Kit (eBioscience). The assay was run on FACSCanto II™ (BD Biosciences). Analyses were performed using FlowJo Software, Tree Star Inc. (Ashland, OR, USA).

**Determination of the Anti-CII-specific IgG Antibodies**

For quantification of anti-CII antibodies in serum, 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 10 μg/ml of native chicken CII (Sigma-Aldrich AB). The samples were serially diluted (1:250, 1:750, 1:2250, 1:6750) in 0.5% bovine serum albumin (BSA) (Sigma-Aldrich AB) in PBS. Biotinylated Fabα2 fragments of goat anti-mouse IgG (Jackson Immuno Research Laboratories, Suffolk, England) were used as secondary antibody. Antibody was performed using horseradish peroxidase 0.5 μg/ml and 2.5 μmol of the enzyme substrate 2,2-azino-bis(3-ethylbenzothiazoline sulfonic acid) (Sigma-Aldrich AB) per ml in citrate buffer (pH 4.2), containing 0.0075% H₂O₂. The absorbance was measured at 405 nm on Spectra Max 340PC (Molecular Devices, Sunnyvale, CA, USA).
Statistical Analysis
The levels of IL-10 in supernatants after treatment with LNT-GFP or LNT-IL-10 before and after LPS stimulation were compared using Two-way ANOVA (GraphPad Prism, GraphPad software, San Diego, CA, USA). All other statistical analysis between independent groups were calculated using the non-parametric Mann-Whitney U-test (GraphPad Prism) as described in the figure legends. A P-value ≤0.05 was regarded as being statistically significant.

Supporting Information

Figure S1 Integration of lentiviral vectors and IL-10 production in vitro. (A) The protein level of IL-10 in supernatants 9 days after infection of HS6 in transduction with LNT-GFP or LNT-IL-10 at MOI 0, 40 or 80 and with or without LPS stimulation. (B) Integration of lentiviral vectors in bone marrow, spleen and synovial cells. The quantification of the lentiviral particles LNT-GFP or LNT-IL-10 are expressed per 100 bone marrow cells, splenocytes or synovial cells. Data in figure 1A were analysed by Two-way ANOVA and data in figure 2B were analysed by Mann-Whitney U-test. Closed circles and black bars represent LNT-GFP and open circles and white bars LNT-IL-10 mice.

Figure S2 Gating strategy for detecting IL-10 expression in CD19+MHCII+ B cells using flow cytometry.

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Author Contributions
Conceived and designed the experiments: IG LH. Performed the experiments: LH TE PJ IG ST UL. Analyzed the data: LH TE IG WvdB.

Contributed reagents/materials/analysis tools: UL. Wrote the paper: LH TE IG WvdB.

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