Systemic overexpression of interleukin-22 induces the negative immune-regulator SOCS3 and potently reduces experimental arthritis in mice

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Abstract

Objective. High levels of IL-22 are present in serum and synovial fluid of patients with RA. As both pro- and anti-inflammatory roles for IL-22 have been described in studies using animal models of RA, its exact function in arthritis remains poorly defined. With this study we aimed to further unravel the mechanism by which IL-22 exerts its effects and to decipher its therapeutic potential by overexpression of IL-22 either locally or systemically during experimental arthritis.

Methods. CIA was induced in DBA-1 mice by immunization and booster injection with type II collagen (col II). Before arthritis onset, IL-22 was overexpressed either locally by intra-articular injection or systemically by i.v. injection using an adenoviral vector and clinical arthritis was scored for a period of 10 days. Subsequently, joints were isolated for histological analysis of arthritis severity and mRNA and protein expression of various inflammatory mediators was determined in the synovium, spleen and serum.

Results. Local IL-22 overexpression did not alter arthritis pathology, whereas systemic overexpression of IL-22 potently reduced disease incidence, severity and pathology during CIA. Mice systemically overexpressing IL-22 showed strongly reduced serum cytokine levels of TNF-α and macrophage inflammatory protein 1α that correlated significantly with the enhanced expression of the negative immune regulator SOCS3 in the spleen.

Conclusion. With this study, we revealed clear anti-inflammatory effects of systemic IL-22 overexpression during CIA. Additionally, we are the first to show that the protective effect of systemic IL-22 during experimental arthritis is likely orchestrated via upregulation of the negative regulator SOCS3.

Key words: cytokines, inflammation, experimental arthritis, rheumatoid arthritis, IL-22

Introduction

RA is an autoimmune disease of unknown aetiology that is characterized by chronic inflammation of the joints leading to destruction of articular cartilage and bone. Although the disease pathogenesis is much better understood, the exact role of some mediators still needs to be further unravelled. IL-22 was first described in mice as the IL-10-related T cell–derived inducible factor α (IL-TIFα). This cytokine is a member of the IL-10 family and shows 22% amino acid identity with IL-10 [1, 2]. Moreover, murine IL-22 strongly resembles its human ortholog with an amino acid similarity of 78% [3]. Major IL-22-producers are the Th17 cells, CD4⁺ T cells that are characterized by their production of IL-17 [4]. Additionally, Th1 cells [5], Th22 cells [6, 7], γδ T cells [8, 9], NK cells [10, 11], innate lymphoid cells type 3 (ILC3) [10, 12], macrophages [13] and dendritic cells [14], among others, have been shown to produce IL-22. Target cells of IL-22 are mostly of the non-hematopoietic lineage, like epithelial and stromal cells [15]. Responses to IL-22 are initiated by binding of the cytokine to its receptor complex consisting of an IL-22 receptor 1 (IL-22R1) and an IL-10 receptor 2 (IL-10R2) subunit, the latter being shared with family member IL-10 [16]. While the IL-10R2 subunit is ubiquitously expressed, expression of the IL-22R1 subunit is mainly restricted to non-hematopoietic cells [17]. In addition to this membrane-bound receptor complex, a soluble IL-22 binding protein (IL-22BP) has been identified. This protein binds IL-22, thereby neutralizing the activity of this cytokine [18, 19].
Increased levels of IL-22 have been observed in the serum of patients with RA as compared with healthy controls [20–22] and in RA synovial fluid as compared with that of OA patients [21]. The increased IL-22 serum level in RA patients is correlated with multiple clinical disease parameters, like the 28-joint DAS [22], serum levels of RF [21, 22] and ACPA [21]. Furthermore, bone erosions were shown to be more severe in patients with high IL-22 serum levels [20–22]. The role of IL-22 has been studied extensively in animal models for various auto-inflammatory diseases. In animal models for IBD, IL-22 was shown to play a protective role. Overexpression of IL-22 led to reduced intestinal inflammation [23, 24], whereas blocking of IL-22 aggravated the disease [25]. In contrast, neutralization of IL-22 ameliorated disease development in a murine psoriasis-like skin inflammation model, while administration with recombinant IL-22 induced development of this inflammatory skin disease [26]. In an animal model for airway inflammation, blocking IL-22 ameliorated disease development. Interestingly, mice also deficient for IL-17 showed exacerbated airway inflammation, suggesting that in the absence of IL-17, IL-22 is protective in this model [27]. Interestingly, reported data describing the role of endogenous IL-22 in animal models for RA showed mostly a pro-inflammatory role for IL-22 [28–32].

We aimed to decipher this pathogenic potential of IL-22 during experimental arthritis by adenoviral overexpression either locally or systemically. We further aimed to unravel the mechanism by which IL-22 exerts its effects during CIA. While most studies focussing on IL-22 in experimental arthritis used IL-22-targeting antibodies or recombinant proteins, we were interested in using a different approach: investigating the effect of IL-22 on disease development and progression with increased levels of local or systemic IL-22 in CIA by using adenoviral expression vectors. One of the major advantages of adenoviral vectors is that they provide the most efficient in vivo gene transfer, leading to high transgene expression within hours after injection compared with other viral vector systems and target a wide variety of cell types, both quiescent and proliferating cells [33]. Also intravenously injected adenoviruses are easily taken up by the spleen [34]. Based on the observation that IL-22 levels correlate with disease parameters in RA patients, and that in animal models of arthritis IL-22 was predominantly pro-inflammatory, we hypothesized that overexpression of IL-22 would aggravate arthritis. However, during this study we showed that locally overexpressed IL-22 did not alter CIA pathology, whereas systemically overexpressed IL-22 resulted in anti-inflammatory effects on CIA that were accompanied by upregulation of the negative immune-regulator suppressor of cytokine signalling 3 (SOCS3).

### Methods

#### Mice

Female DBA/1JRj mice and C57BL6N were purchased from Janvier-Elevage (Le Genest Saint Isle, France). Animals were used between 10 and 12 weeks of age unless otherwise specified and were housed under specific pathogen-free conditions until transfer to individually ventilated cage units after injection with adenoviral constructs. A standard diet and water were provided ad libitum. All animal procedures were approved by the ethics committee of the Radboud University, Nijmegen, The Netherlands (permit RU-DEC 2013-014).

#### Induction of CIA

CIA was induced as described previously in DBA/1JRj mice [35]. Arthritis development was macroscopically scored three times a week using an arbitrary scoring system of 0–2 per paw and 0–8 per mouse, according to changes in redness and/or swelling of the paws. After 10 days the mice were anesthetized using 2–3% isoflurane to collect serum before being sacrificed by cervical dislocation.

#### Induction of K/BxN serum transfer-induced arthritis (STIA)

K/BxN T cell receptor–transgenic mice on a C57BL/6 background (K/B) were a kind gift from Christophe Benoist and Diane Mathis (Harvard Medical School, Boston, MA, USA). By crossing K/B mice with NOD/Lt animals, arthritic K/BxN mice were obtained in the Nijmegen animal facility (permit RU-DEC 2011-187). A total of 150 μl of serum derived from those mice was injected i.p. in C57BL/6N mice on days 0 and 2. On days 4 and 7 of the experiment, the knee joints were isolated for histological analysis.

#### Induction of streptococcal cell wall arthritis (SCW)

Streptococcus pyogenes T12 organisms were cultured overnight in Todd–Hewitt broth. Cell wall fragments were prepared as described previously [36]. Arthritis was subsequently induced by injecting 25 μg SCW
fragments intra-articularly (i.a.) in C57BL/6N mice, resulting in acute local inflammation. Mice were sacrificed on day 7 and knee joints were isolated for histological analysis.

Adenoviral vectors

The adenoviral expression vector for murine IL-22 (AdIL-22) was kindly provided by J.K. Kolls (Tulane University School of Medicine, New Orleans, LA, USA) and the control adenoviral expression vector for luciferase (AdLuc) was generated in-house. All viruses are replication deficient and have the transgene under control of a CMV promoter. Twenty-one days after immunization with CII, 1 x 10⁷ plaque-forming units (PFU) of adenoviruses were injected i.a. into both knee joints for local overexpression, or 3 x 10⁸ PFU of adenoviruses were injected i.v. via the orbita plexus for systemic overexpression of SCW arthritis or STIA. A total of 1 x 10⁷ PFU of adenoviruses were injected i.a. in naïve mice or 1 day prior to induction of SCW arthritis or STIA.

Histology

Isolated joints were fixed for at least 4 days in 4% formaldehyde, decalcified in 5% formic acid for 7 days at room temperature and subsequently dehydrated and embedded in paraffin. Standard frontal sections of 7 µm were mounted on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany). Haematoxylin and eosin (H&E) staining and Safranin O (SO) staining were performed to study pathologic features of the joint. The severity of arthritis in the joints was scored on an arbitrary scale of 0–3, where 0 was no pathology and 3 was maximal pathology, as previously described for three different parameters [joint inflammation, proteoglycan (PG) depletion from the cartilage matrix and bone erosion] [37]. Histopathological changes were scored on three serial sections of the joint, spaced 140 µm apart, in a blindfolded manner.

RNA isolation and quantitative real-time PCR

For RNA isolation of the synovial tissue, lateral and medial pieces were punched out from synovium surrounding the patella using a 3 mm biopsy punch (Stiefel, Wächtersbach, Germany). The synovial punches were pooled per knee joint and immediately frozen in liquid nitrogen. Subsequently the punches were disrupted and homogenized using the MagNA Lyzer (Roche, Basel, Switzerland). Total RNA was isolated using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer’s protocol. After isolation, RNA was treated with DNase and reverse transcribed into complementary DNA (cDNA) as previously described [32]. Gene expression levels were determined by quantitative PCR on the StepOnePlus sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green technology (Applied Biosystems) and 2 µM primers (Biolegio, Nijmegen, The Netherlands). Specific gene expression was normalized for expression of the reference gene Gapdh. To allow for statistical analysis, the −ΔCt value of samples with mRNA levels below the detection limit (≥40 cycles) was set at −25. Primer sequences can be found in Supplementary Table S1, available at Rheumatology online.

Cytokine and chemokine measurements

Cytokine levels were determined using the Luminex multi-analyte technology on the Luminex 200 (Bio-Rad, Hercules, CA, USA) in combination with BioRad cytokine kits 1:4 diluted in serum diluent according to the manufacturer’s protocol. To determine the levels of cytokines in synovial washouts, patellae with surrounding soft tissue consisting of the tendon and synovium were dissected in a standardized manner. Patellae were cultured in RPMI 1640 medium containing 0.1% BSA (200 µl/patella) for 1 h at room temperature. Thereafter, supernatant was harvested and centrifuged for 5 min at 1000 g. Cytokine levels were determined using the Luminex multi-analyte technology in 50 µl of synovial washout medium.

Statistical analysis

To determine the level of statistical significance between means of experimental groups, the t test or a one- or two-way analysis of variance (ANOVA) was used unless stated otherwise. This depended on the number of experimental groups and normality testing using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA). P-values <0.05 were considered significant.

Results

Local IL-22 overexpression did not alter CIA joint pathology

After demonstrating that intra-articular injection of AdIL-22 resulted in overexpression of IL-22 in a naïve joint (Supplementary Fig. S1A, available at Rheumatology online), we overexpressed IL-22 locally during CIA. IL-22 levels were not monitored over time, but interestingly we could still detect elevated levels of IL-22 in the serum of mice 10 days after i.a. injection with AdIL-22 (Supplementary Fig. S1B, available at Rheumatology online). Local IL-22 overexpression during CIA did not affect synovial mRNA expression or protein serum levels of various pro- and anti-inflammatory markers, at least not detectable 10 days after virus injection (data not shown, performed by Roeleveld et al.). Macroscopic scoring showed that arthritis incidence was not affected by local IL-22 overexpression (Fig. 1A). When analysing the arthritis severity in the mice that did develop arthritis, the total arthritis score of the four paws was only significantly increased by IL-22 overexpression at day 28 of CIA (Fig. 1B). No difference in arthritis severity was observed when only scoring the front legs (Fig. 1C). Interestingly, when only the hind paws, of which the
Ipsilateral knee joints both received the virus injections, were included in the analysis, overexpression of IL-22 slightly increased the severity of clinical arthritis at days 28 and 31, indicating a local pro-inflammatory role for IL-22 (Fig. 1D).

By subsequent histological analysis, we aimed to unravel the effect of local IL-22 overexpression on arthritis severity in more detail. In naïve mice, IL-22 overexpression induced short-term upregulation of IL-6, Keratinocyte Chemoattractant (KC), S100A9 and receptor activator of nuclear factor κB ligand (RANKL) (Supplementary Fig. S2A, available at Rheumatology online), although this was not sufficient to induce joint pathology (Supplementary Fig. S2B and C, available at Rheumatology online). Based on this pro-inflammatory response and the macroscopic scores of CIA at the time of sacrifice (day 31), we expected local IL-22 overexpression to aggravate synovial inflammation and joint destruction. However, as demonstrated in Fig. 2A and B, arthritis pathology at day 31 of CIA showed no differences between AdIL-22- and AdLuc-injected knee joints on various histological parameters (Fig. 2A and B). Additionally, no effects of IL-22 overexpression were found in the ipsilateral ankles (Supplementary Fig. S3, available at Rheumatology online). To exclude that the severity of the CIA model overruled the pro-inflammatory effects of IL-22 overexpression, we also applied local AdIL-22 during the immune complex–dependent K/BxN serum transfer–induced arthritis and the toll-like receptor 2–driven SCW arthritis. However, local IL-22 overexpression did not affect joint pathology in these arthritis models either (Supplementary Figs S4 and S5, available at Rheumatology online).

Systemic IL-22 overexpression potently reduced arthritis pathology

In addition to the effect of local IL-22 overexpression during experimental arthritis, we investigated the potential of IL-22 to enhance CIA by systemic administration of AdIL-22. Our group previously used a similar approach to demonstrate the pro-inflammatory and destructive effects of IL-17 [38]. Surprisingly, development of CIA was delayed in mice injected with AdIL-22 and...
arthritis incidence at the end of the study was clearly reduced compared with the AdLuc-injected mice (CIA incidence of 83% in AdLuc vs 50% in AdIL-22; Fig. 3A). When studying arthritis severity, strikingly and significantly reduced macroscopic scores were detected in mice receiving systemic AdIL-22 as compared with the AdLuc-injected control group (Fig. 3B). In line with macroscopic inflammation, histological analysis showed significantly reduced cellular influx into the ankle joints of mice injected with AdIL-22 (Fig. 3C and D). Additionally, cartilage PG depletion from these ankles was dramatically reduced (Fig. 3C and D) and small but significant protective effects on bone erosion were observed (Fig. 3C). Only the mice that developed arthritis were used in the analysis.

**Systemic IL-22 overexpression reduces expression of various inflammatory mediators**

To investigate whether the anti-inflammatory effect of IL-22 overexpression during CIA was accompanied by reduced serum cytokine levels, a multi-analyte Luminex analysis was performed. Highly elevated levels of IL-22 were detected 10 days after systemic IL-22 overexpression in CIA mice (Fig. 4), up to 200 times higher than after local overexpression of IL-22 (Supplementary Fig. S1B, available at Rheumatology online). In line with macroscopic inflammation, histological analysis showed significantly reduced cellular influx into the ankle joints of mice injected with AdIL-22 (Fig. 3C and D). Additionally, cartilage PG depletion from these ankles was dramatically reduced (Fig. 3C and D) and small but significant protective effects on bone erosion were observed (Fig. 3C). Only the mice that developed arthritis were used in the analysis.

Systemic IL-22 overexpression induces splenic SOCS3 mRNA expression

To explain the anti-inflammatory effect of systemic IL-22 overexpression, we studied the effect of AdIL-22 on the expression of the anti-inflammatory protein SOCS, an important negative regulator of the immune system [40]. Interestingly, a strong and significant upregulation of SOCS3 was detected in the spleen, but not synovium,
of mice after systemic overexpression of IL-22 during CIA, whereas SOCS1 levels were not significantly affected (Fig. 5A and B).

Interestingly, a significant negative correlation between splenic SOCS3 mRNA expression and serum levels of TNF-α (Fig. 6A) and MIP-1α (Fig. 6B) was detected, suggesting that the observed reduction in serum cytokine levels is mediated in a SOCS3-dependent manner. No significant correlation was observed for IFN-γ (Fig. 6C) and IL-10 (Fig. 6D).

Discussion

Despite the numerous animal studies performed to delineate the dual role of IL-22 in the development and progression of experimental arthritis, no consensus on this subject has been reached. While most studies focussing on IL-22 in experimental arthritis used IL-22-targeting antibodies or recombinant proteins, we were interested in using a different approach: investigating the effect of IL-22 in CIA by using adenoviral expression vectors. One of the major advantages of adenoviral vectors is that they provide the most efficient in vivo gene transfer, leading to high transgene expression within hours after injection, and target a wide variety of cell types, both quiescent and proliferating cells [33]. Adenoviral vectors are the most commonly used in clinical trials worldwide and account for >20% of all gene therapy trials [41]. Intravenously injected adenoviruses are easily taken up by the spleen and show gene expression by antigen-presenting cells [34], whereas intra-articularly injected adenoviruses target mostly the fibroblasts in the synovial lining layer [42]. In our studies we showed that local IL-22 overexpression during CIA did not alter joint pathology, whereas systemic overexpression of IL-22 dramatically reduced the development of
arthritis, which was accompanied by upregulation of SOCS3.

Unexpectedly, local adenoviral IL-22 overexpression did not induce joint pathology in naïve mice, nor did it aggravate the severity of innate immune-driven arthritis in mice with K/BxN serum transfer– or SCW-induced arthritis. Although it caused a rapid upregulation of pro-inflammatory cytokines and pro-destructive mediators, we also observed that this upregulation was already greatly diminished after 24 h, while the expression of IL-22 peaked at this time point. This discrepancy suggests that the early pro-inflammatory effects of IL-22 may be subsequently inhibited by negative feedback mechanisms, probably lasting too short to induce or aggravate joint pathology.

Various publications describe a predominantly pro-inflammatory phenotype for IL-22 during adaptive immune-driven experimental arthritis. Blocking IL-22 suppressed arthritis in arthritic IL-1Ra−/− mice [32]. Additionally, another study showed reduced severity of CIA in IL-22−/− mice on a C57BL/6 background. However, no effects of IL-22 deficiency on disease incidence were detected [30]. Another study showed a protective role for endogenous IL-22 before the onset and a pathogenic role after the onset of CIA in DBA1- or IFN-γ-deficient mice, and the pathogenic effect of IL-22 was suggested to be dependent on suppression of IFN-γ responses [43].

Based on these studies on downregulating IL-22 signalling, we expected that a systemic overexpression of IL-22 would cause an aggravation of CIA development. However, a highly significant reduction in CIA incidence and severity was observed in our study. This protective effect of IL-22 overexpression during CIA was confirmed by an independent observation in a similar study design (personal communication, F. Apparailly, INSERM U844, France). The reduced CIA pathology observed during systemic IL-22 overexpression was accompanied by suppressed B cell responses as demonstrated by lower IgG2a serum levels.

Intriguingly, the therapeutic effect of systemic IL-22 overexpression and lack of effects upon local injection were similar to those previously reported by our group on adenoviral overexpression of SOCS3 [44]. SOCS3 negatively regulates various cytokine signalling pathways [40]. Whereas local SOCS3 overexpression during CIA did not protect from arthritis development, systemic

**Fig. 4** Serum protein levels of IL-22, IFN-γ, TNF-α, MIP-1α and IL-10

![Protein level serum](image)

Mice were injected intravenously with AdIL-22 or AdLuc virus after receiving col II booster at day 21. Mice were sacrificed 10 days after virus injection and serum was collected to determine protein levels using Luminex (n = 12 mice/group; t test). *P < 0.05, **P < 0.01, ***P < 0.001. Values are mean (S.E.M.).

**Fig. 5** Splenic SOCS3 mRNA expression increased after systemic IL-22 overexpression

![SOCS3 mRNA expression](image)

Mice with CIA were sacrificed 10 days after AdIL-22 or AdLuc virus injection (day 31 of CIA). Subsequently, (A) splenic tissue and (B) synovium punches were isolated and relative mRNA expression of SOCS genes was determined using quantitative PCR. N = 4–6 mice/group. **P < 0.01; unpaired t test. Values are mean (S.E.M.).
SOCS3 overexpression resulted in the generation of tolerogenic antigen-presenting cells, impairing antigen-specific T cell activation and thereby suppressing arthritis [44]. Explaining the protective effects of IL-22 through SOCS3 induction could make sense, as IL-22 mainly signals through STAT3 [45], which may in turn lead to the activation of a negative feedback mechanism via SOCS3 [46]. The fact that IL-22 has been shown to induce SOCS3 expression in lung epithelial [46] and liver cells [47] leads to the hypothesis that a similar mechanism may apply during CIA. Indeed, a significant increase in SOCS3 mRNA expression was detected in splenocytes, but not synovocytes, from AdIL-22-injected mice.

In line with the increased SOCS3 levels, we observed a significant reduction in IFN-γ, TNF-α, MIP-1α and IL-10 serum levels in mice upon systemic IL-22 overexpression during CIA. This typical cytokine profile suggests that the anti-inflammatory effect of IL-22 mainly affects cytokine production by monocytes/macrophages, although IL-22R is mainly expressed by non-hematopoietic cells, suggesting an indirect effect of IL-22 on these cytokines [17]. The increased SOCS3 expression in splenocytes negatively correlated with the reduced serum levels of TNF-α and MIP-1α, suggesting that the observed anti-inflammatory effect of systemic IL-22 overexpression is mediated via a SOCS3-dependent pathway. In synovial tissue, local IL-22 did not induce a detectable upregulation of SOCS3 expression. Probably only high doses of IL-22 induce SOCS3. Local IL-22 levels were approximately 6-fold lower than systemic levels at day 10 after intra-articular or systemic overexpression. Another explanation could be the route of administration and the target cells reached, as only intravenously injected AdIL-22 resulted in arthritis protection and increased SOCS3 expression in the spleen, suggesting that this is a systemic process.

Even though our IL-22-induced SOCS3 concept explains our current data and probably more studies using overexpression of IL-22, it cannot fully account for historic data on the dual role of IL-22. The IL-22-induced responses appear to depend on whether the cytokine is endogenously expressed or exogenously administered: whereas endogenous IL-22 plays a pro-inflammatory role in arthritis, exogenous (high) levels of IL-22 have been demonstrated to be anti-inflammatory in experimental arthritis models. Additionally, it seems that the location of recombinant protein/viral vector injection is important for the outcome of the intervention. In general, studies blocking endogenous IL-22 using systemically applied neutralizing antibodies or genetically modified mice show reduced development of experimental arthritis [28–32, 48], indicating a pro-

**Fig. 6** Splenic SOCS3 expression negatively correlates with serum levels of TNF-α and MIP-1α.

Relative splenic SOCS3 mRNA expression ($2^{-\Delta\Delta CT} \times 100$) was plotted against serum levels of (A) TNF-α, (B) MIP-1α, (C) IFN-γ and (D) IL-10. $N = 12$ mice. Pearson r correlation.
of IL-22 in arthritis. With these studies identifying SOCS3 induction by IL-22 associated with increased gene regulation of SOCS3. Finally, the inflammatory context seems to affect the magnitude of the IL-22 effects on experimental arthritis: neutralization of the cytokine before arthritis onset potently reduces disease severity, whereas these effects have been described as absent when neutralizing IL-22 during later stages of arthritis development [48].

To date, both pro- and anti-inflammatory roles have been described for IL-22, making it a debatable target in the treatment of RA. However, as only high, non-physiological concentrations of exogenous IL-22 were demonstrated to be anti-inflammatory, and IL-22 was shown to drive the local arthritis process using blocking and knockout studies, therapeutic targeting of endogenous IL-22 is expected to be beneficial without risk of adverse inflammatory responses, contributing to reduced pathology and symptoms in the treatment of RA. With these studies we revealed a subtle pro-inflammatory effect of IL-22 after local overexpression. More importantly, a clear anti-inflammatory effect of systemic IL-22 after local overexpression. More importantly, a clear anti-inflammatory effect of systemic IL-22 overexpression during CIA was described that was associated with increased gene regulation of SOCS3. With these studies identifying SOCS3 induction by IL-22, we have contributed to understanding the dual role of IL-22 in arthritis.

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Supplementary data

Supplementary data are available at Rheumatology online.

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