

EXTENDED REPORT

Anti-inflammatory effects of cell-based therapy with tyrosine hydroxylase-positive catecholaminergic cells in experimental arthritis

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ABSTRACT

Objectives Studies in rheumatoid arthritis (RA), osteoarthritis (OA) and mice with arthritis demonstrated tyrosine hydroxylase-positive (TH⁺) cells in arthritic synovium and parallel loss of sympathetic nerve fibres. The exact function of TH⁺ cells and mode of TH induction are not known.

Methods Synovial cells of RA/OA were isolated and cultured under normoxic/hypoxic conditions with/without stimulating enzyme cofactors of TH and inhibitors of TH. We studied TH expression and release of cytokines/catecholamines. In vivo function was tested by cell therapy with TH⁺ neuronal precursor cells (TH⁺ neuronal cells) in DBA/1 mice with collagen type II-induced arthritis (CIA).

Results Compared with normoxic conditions, hypoxia increased TH protein expression and catecholamine synthesis and decreased release of tumour necrosis factor (TNF) in OA/RA synovial cells. This inhibitory effect on TNF was reversed by TH inhibition with α -methyl-para-tyrosine (α MPT), which was particularly evident under hypoxic conditions. Incubation with specific TH cofactors (tetrahydrobiopterin and Fe²⁺) increased hypoxia-induced inhibition of TNF, which was also reversed by α MPT. To address a possible clinical role of TH⁺ cells, murine TH⁺ neuronal cells were generated from mesenchymal stem cells. TH⁺ neuronal cells exhibited a typical catecholaminergic phenotype. Adoptive transfer of TH⁺ neuronal cells markedly reduced CIA in mice, and 6-hydroxydopamine, which depletes TH⁺ cells, reversed this effect.

Conclusions The anti-inflammatory effect of TH⁺ neuronal cells on experimental arthritis has been presented for the first time. In RA/OA, TH⁺ synovial cells have TH-dependent anti-inflammatory capacities, which are augmented under hypoxia. Using generated TH⁺ neuronal cells might open new avenues for cell-based therapy.

INTRODUCTION

During the last two decades, neuroendocrine-immune interactions became more and more relevant in understanding pathophysiology of rheumatic diseases.^{1–5} For example, the actual target of inflammation in rheumatoid arthritis (RA), the synovial tissue, is strongly affected by the sympathetic nervous system depending on the phase of inflammation. In the early acute phase of experimental arthritis, the sympathetic nervous system has a pro-inflammatory role,^{6–8} whereas in the late

chronic phase anti-inflammatory effects have been described.⁸ At the beginning of the chronic phase, the loss of sympathetic nerve fibres in synovial tissue was reported, which is paralleled by appearance of tyrosine hydroxylase-positive (TH⁺) catecholamine-producing cells.^{9–12} Recently, these TH⁺ cells of RA and osteoarthritis (OA) patients have been shown to exhibit anti-inflammatory properties in vitro.⁹ However, important cofactors and inducers of this catecholamine-producing enzyme have never been studied and the role of TH⁺ cells in vivo is not known yet.

TH is regulated by the phosphorylation status of subunits,^{13–14} environmental pH value,¹⁵ specific stimulatory cofactors such as tetrahydrobiopterin (BH₄) and bivalent iron (Fe²⁺),^{13–16} product feedback inhibition, and proteasomal degradation.¹⁷ In addition, hypoxia was shown to enhance TH gene expression, TH mRNA stability and TH activity by increasing phosphorylation of TH.^{18–19} Inflammation is accompanied by hypoxia;²⁰ hypoxia plays an important role in disease progression in RA.^{21–23} Under these considerations, we expected that hypoxia influences expression and activity of TH in chronically inflamed tissue. This might result in enhanced catecholamine synthesis of TH⁺ cells, which in turn might affect inflammatory cytokine production in vitro.

In order to study TH⁺ cell effects in vivo, treatment of arthritic animals with TH⁺ cells is mandatory. To our knowledge, TH⁺ cells do not express suitable surface markers to separate these cells by respective sorting techniques demanding a different strategy. Human TH⁺ cells expressing specific markers such as TH, β -III-tubuline (neuronal intracellular scaffold protein), vesicular monoamine transporter 2 (VMAT-2) and nuclear receptor related 1 (Nurr1) protein have been generated on the basis of mesenchymal stem cells (MSCs).^{24–25} These cells are TH⁺ neuronal precursor cells (called TH⁺ neuronal cells). However, no similar differentiation experiments have been performed to generate TH⁺ neuronal cells in rodents, and these cells have not been used in experimental arthritis in mice or in other disease models.

The aim of these studies was to investigate hypoxia-induced effects on TH expression, catecholamine release and inflammatory cytokine release in RA/OA human synovial cells in vitro. TH cofactors (BH₄ and Fe²⁺) were used to amplify the function of TH, and cytokine release was



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investigated. In addition, TH⁺ neuronal cells were generated from murine MSCs in order to perform adoptive transfer experiments during collagen type II-induced arthritis (CIA) in mice. Effects of adoptively transferred TH⁺ neuronal cells were studied under treatment with 6-hydroxydopamine (6OHDA) to kill TH⁺ cells.

MATERIALS AND METHODS

Human samples were collected with informed consent of the involved individuals, and mouse experiments were performed in accordance with local institutional and governmental regulations for animal use. The project was approved by the Ethics Committee of the University of Regensburg. A more complete and detailed description of the methods is included in online supplementary SI materials and methods.

Human synovial cell culture

Human mixed synoviocytes were incubated under hypoxic and normoxic conditions for 24 h with specific TH cofactors BH₄ (6R-5,6,7,8-tetrahydrobiopterin dihydrochloride), bivalent iron (Fe²⁺ sulfate heptahydrate), α -methyl-para-tyrosine (α mpT; a competitive TH blocker), monoaminooxidase (MAO) inhibitor bifemelane hydrochloric acid (HCL) and catechol-O-methyltransferase (COMT) inhibitor OR-486 at indicated concentrations. Supernatant was collected and concentrations of interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF) were determined by ELISA or Luminex technology. For catecholamine determination high pressure liquid chromatography (HPLC) was

performed. Expression of TH was analysed by immunocytochemistry in chamber slides and via western blotting.

Mouse experiments

Murine bone marrow-derived stem cells (MSCs) were isolated from bone chips of DBA/1J mice, expanded and characterised. Catecholaminergic induction was initiated with a specific catecholaminergic cocktail. Generated TH⁺ neuronal cell characterisation was performed by light and immunofluorescence microscopy, western blotting, HPLC and using 6OHDA, which destroys TH⁺ cells specifically. For adoptive transfer, TH⁺ neuronal cells were intravenously injected into the tail vein of DBA/1J mice with collagen-induced arthritis when an arthritis score of 10 was reached. Clinical arthritis score was determined until day 55 after immunisation. In another experiment, catecholaminergic cells were labelled using the red fluorescent PKH26 in order to characterise localisation of cells in the body of mice. Sections of organs and paws were screened for labelled cells by fluorescence microscopy. In a further set of experiments, beginning on day 10 after TH⁺ neuronal cell adoptive transfer, 6OHDA was injected intraperitoneally on three consecutive days and clinical arthritis score was determined in order to follow-up the role of injected TH⁺ neuronal cells.

RESULTS

Hypoxia induces TH in mixed synovial cells

Hypoxic conditions compared with normoxia induced TH protein expression in both OA and RA cell cultures (figure 1A). This observation was confirmed by western blotting because

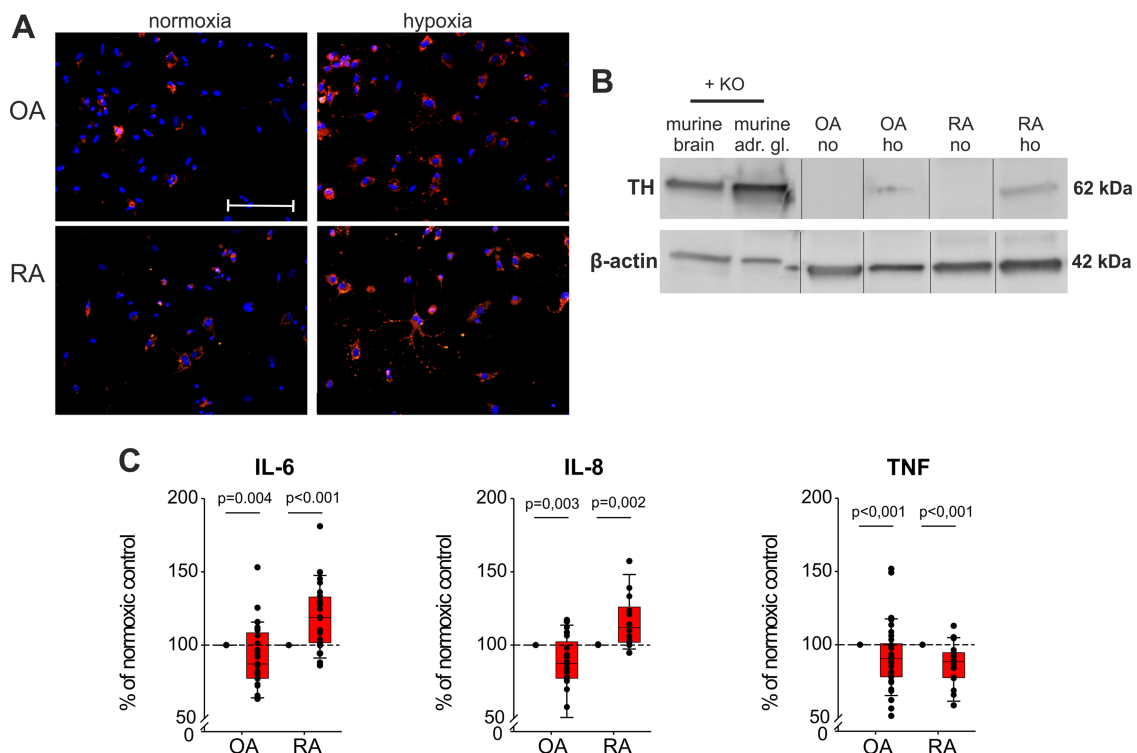


Figure 1 Effects of hypoxia on tyrosine hydroxylase (TH) expression and cytokine release in synovial cells of patients with osteoarthritis (OA) and rheumatoid arthritis (RA). (A) Immunofluorescent detection of TH-positive synovial cells after 24 h of normoxic or hypoxic incubation (Bar 100 μ m). (B) Western blot analysis of TH expression in synovial cells from OA and RA patients after 24 h of normoxic or hypoxic incubation (see also supplementary figure 2). (C) Quantification of released cytokines in supernatants of synovial cells from OA and RA patients after 24 h of normoxic or hypoxic incubation (n=21–44). Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. The red boxes represent hypoxic conditions. Values are demonstrated in per cent of the normoxic control (untreated normoxic control=100%, broken line). adr. gl, adrenal gland; no, normoxia; ho, hypoxia.

after 24 h under hypoxic conditions, total TH was increased compared with normoxic conditions (figure 1B).

Hypoxia-dependent TH induction influences inflammatory cytokine release

Incubation of mixed synovial cells under hypoxic conditions resulted in an altered inflammatory cytokine release pattern compared with normoxic incubation (figure 1C). In OA synovial cells, each analysed cytokine (IL-6, IL-8 and TNF) was significantly reduced by hypoxia (figure 1C). In contrast, in RA synovial cells, IL-6 and IL-8 increased significantly after hypoxic incubation, whereas TNF was significantly reduced similar to OA synovial cells (figure 1C).

Involvement of TH in these observed effects was tested using the competitive TH inhibitor α mpT, which was able to reverse hypoxia-mediated TNF reduction significantly at 1 μ g/mL in both OA and RA synovial cell cultures (figure 2A). In OA synovial cells under normoxic conditions, α mpT at 0.1 μ g/mL also increased TNF (figure 2A). In the case of RA cells, the 1 μ g/mL

concentration of α mpT was able to significantly increase TNF under normoxic conditions (figure 2A). The dose-response curve has a bell-shaped form, which can be due to toxic/unspecific effects at 10 μ g/mL (figure 2A).

Specific TH cofactors enhance hypoxia-mediated effects on cytokines

In order to further increase TH activity, specific TH cofactors such as BH₄ and Fe²⁺ were added. In combination (BH₄ 0.1 mM and Fe²⁺ 10 nM), these cofactors enhanced hypoxia-mediated effects on IL-6 and TNF in both OA and RA synovial cell cultures (figure 2B): In OA cell culture, IL-6 decreased from 85% of the normoxic control to 60% and TNF declined from 90% to 80%. In RA synovial cells, IL-6 rose from 110% of the normoxic control to 140% and TNF decreased from 85% to 75% (figure 2B). In OA synovial cells, 0.1 mM BH₄ and 10 nM Fe²⁺ exhibited inhibitory effects on IL-6 also under normoxia (figure 2B). All these described effects caused by BH₄ and Fe²⁺ were reversed by the competitive TH inhibitor α mpT (figure 2B).

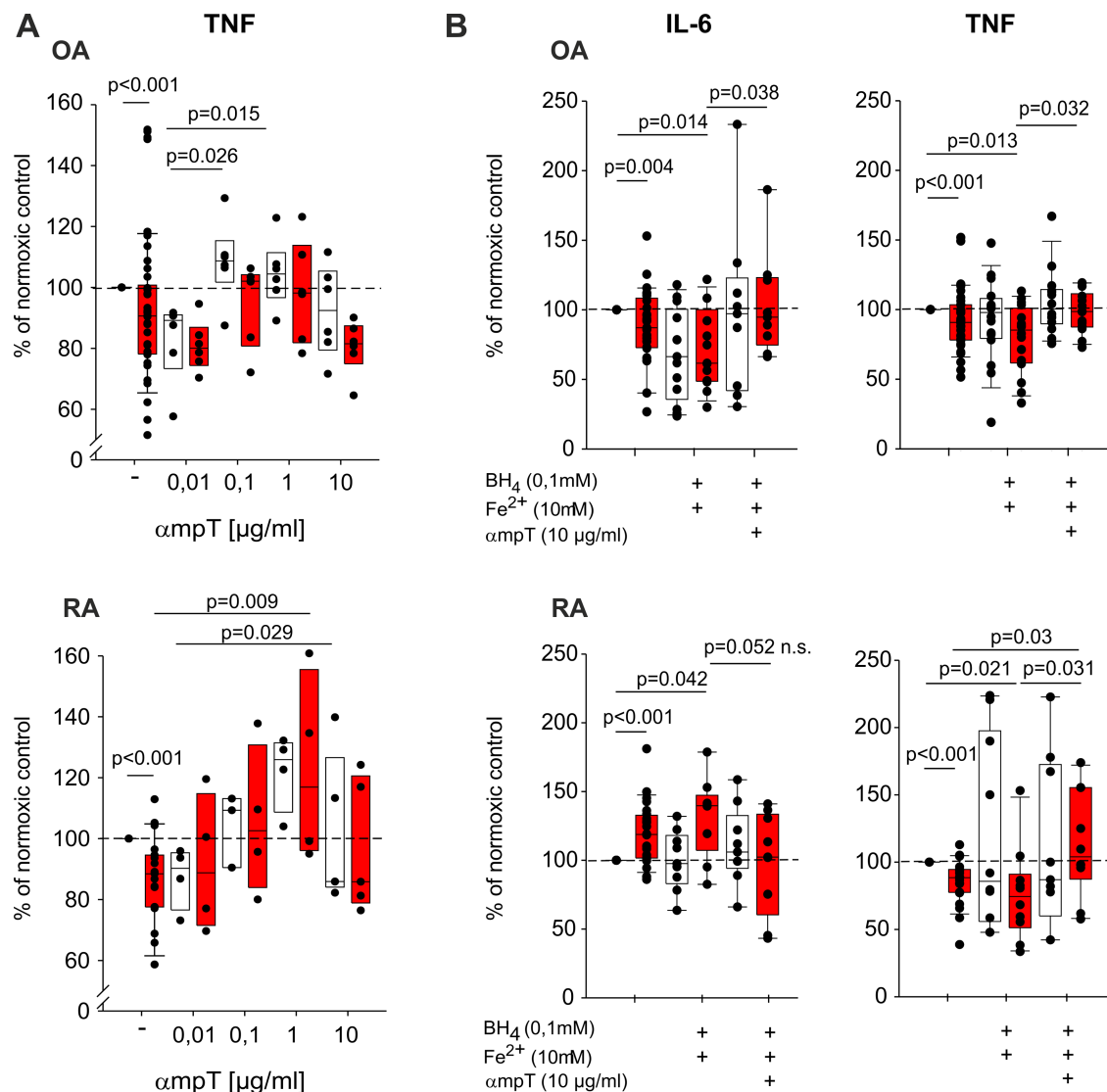


Figure 2 Modulation of inflammatory cytokine release of synovial cells by tyrosine hydroxylase (TH) cofactors and a TH inhibitor in synovial cells of patients with osteoarthritis (OA) and rheumatoid arthritis (RA). (A) Reversion of hypoxia-mediated TNF inhibition by the competitive TH inhibitor α -methyl-para-tyrosine (α mpT, n=6–44). (B) Influence of the two TH cofactors tetrahydrobiopterin (BH₄) and Fe²⁺ on release of IL-6 and TNF with/without α mpT (n=9–10). Box plots are explained in legend to figure 1. The red (white) boxes show hypoxic (normoxic) conditions. Data points are presented as per cent of the untreated normoxic control group (untreated normoxic control=100%, broken line).

Catecholamine biosynthesis in synovial cell culture

The effect of catecholamines on TNF release demonstrated above might be stronger in the presence of inhibitors of catecholamine-degrading enzymes (MAO and COMT inhibitors). Under hypoxic conditions, MAO and COMT inhibitors enhanced TNF inhibition significantly in both OA and RA synovial cell culture (figure 3A). The same MAO and COMT inhibitor-dependent effect was observed under normoxic conditions (figure 3A).

A good indication of TH activity is the release of catecholamines into the supernatant. Using both OA and RA cells, concentration of catecholamines in cell supernatants incubated under normoxia was at the detection limit. Under hypoxia, higher catecholamine levels were measured (approximately 10^{-9} – 10^{-8} M, figure 3B).

The coincubation with MAO and COMT inhibitors resulted in catecholamine accumulation. After 24 h of hypoxic incubation, higher catecholamine concentrations were detected compared with normoxia or to supernatants without MAO and COMT inhibitors in both OA and RA synovial cell cultures (figure 3B).

Catecholaminergic differentiation of murine MSCs

Until now, we were not able to demonstrate a direct anti-inflammatory effect of TH⁺ cells in experimental arthritis because TH⁺ cells cannot be separated by magnetic or fluorescent sorting techniques. Thus, we decided to generate TH expressing cells from MSCs in mice to treat animals with CIA.

Cells from murine bone chips that proliferated until passage 4 exhibited fibroblast-like morphology (figure 4A,B,F) and a MSC-specific surface marker pattern (see online supplementary figure S1). In addition, these cells were able to differentiate into adipocytes (figure 4C), osteocytes (figure 4D) and chondrocytes (figure 4E) in cell lineage-specific differentiation media.

After 12 days of catecholaminergic differentiation, induced TH⁺ neuronal cells were compared with MSCs morphologically and immunocytologically. In contrast to MSCs, induced TH⁺ neuronal cells expressed neuronal precursors and showed neuron-like morphology (figure 4F). Differentiated TH⁺ neuronal cells formed cell-connected rosette-like patterns, which is characteristic for neurogenic differentiation.²⁶ While MSCs were definitely negative for catecholaminergic markers such as

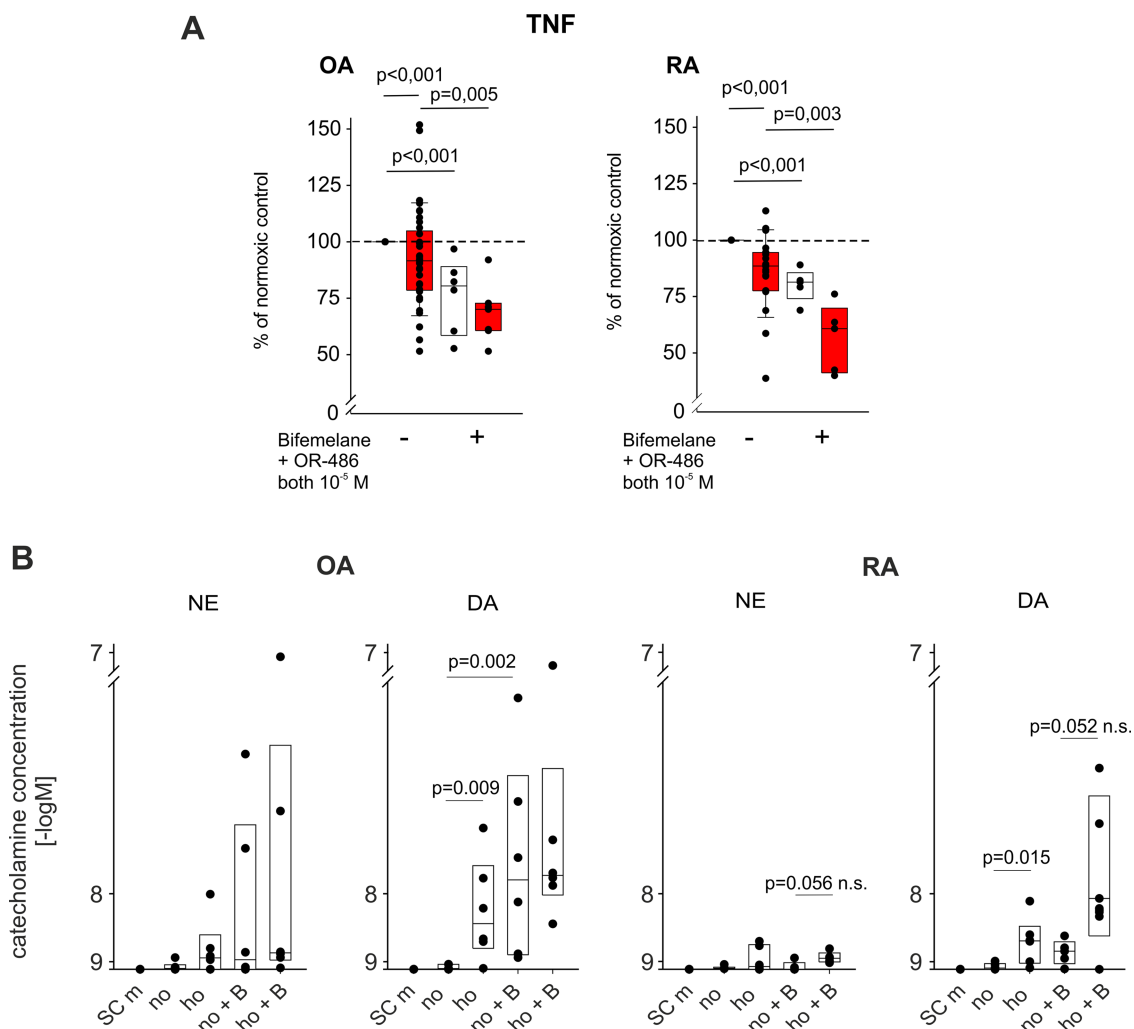


Figure 3 Catecholamine and TNF release of synovial cell cultures of patients with osteoarthritis (OA) and rheumatoid arthritis (RA). Box plots are explained in legend to figure 1. (A) Enhanced TNF inhibition due to catecholamine accumulation caused by treatment of synovial cells with MAO and COMT blockers (each 10^{-5} M; n=6–44). The red (white) boxes in (A) show hypoxic (normoxic) conditions. Values in (A) are demonstrated in % of the normoxic control (untreated normoxic control: 100%, broken line). (B) Catecholamine levels (NE, norepinephrine; DA, dopamine) in synoviocyte supernatants after 24 h of normoxic or hypoxic incubation (n=6–7). SCm, medium without cells; no, normoxia; ho, hypoxia; no+B, normoxia plus blockers of monoamino oxidase (MAO, bifemelane 10^{-5} M) and catechol-O-methyl-transferase (COMT, OR-486 10^{-5} M); ho+B, hypoxia plus MAO and COMT blockers.

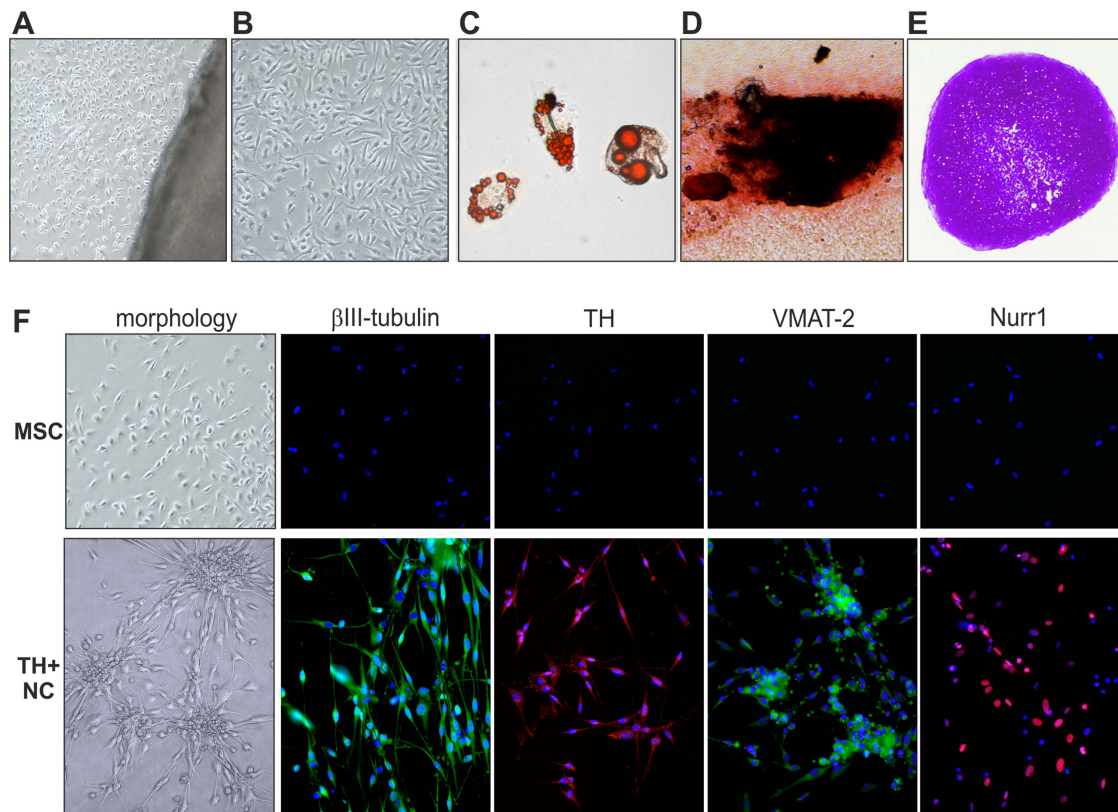


Figure 4 Characterisation of murine mesenchymal stem cell (mMSCs)-derived tyrosine hydroxylase-positive (TH⁺) neuronal cells. (A and B) mMSCs migrating from bone chips (A, magnification 100×) and proliferating in plastic flasks (B, magnification 200×). (C–E) Multilineage differentiation of mMSCs to adipocytes (C, magnification 400×), osteoblasts (D, magnification 100×) and chondrocytes (E, magnification 40×). (F) Characterisation of TH⁺ neuronal cells compared with mMSCs using phase contrast microscopy (200×), and antibodies to βIII-tubulin, TH, vesicular monoamine transporter 2 (VMAT-2), nuclear receptor related 1 (Nurr1). Magnifications: 200×. TH⁺ NC, TH⁺ neuronal cells.

βIII-tubulin, TH, VMAT-2 and Nurr1 (figure 4F), induced TH⁺ neuronal cells clearly expressed all these catecholaminergic markers (figure 4F).

In MSC culture supernatants, catecholamine concentrations were below the detection limit (approximately 10^{-9} M, figure 5A). At day 7 of catecholaminergic differentiation, that is, shortly before brain-derived neurotrophic factor (BDNF) supplementation, cells secreted catecholamines leading to concentrations of up to 10^{-8} M (figure 5A). On day 12, catecholamine concentrations increased to 10^{-7} M (figure 5A). While both norepinephrine and dopamine were measured (figure 5A), epinephrine was not measurable.

In order to study whether 6OHDA can deplete induced TH⁺ neuronal cells in vitro, 6OHDA was incubated together with these cells. After 24 h 6OHDA treatment, no vital induced TH⁺ neuronal cells were detected in chamber slides (figure 5B, red colour=dead cells). In contrast, almost 100% of MSCs survived 6OHDA treatment (figure 5B, green colour=vital cells).

High TH expression of induced TH⁺ neuronal cells was confirmed by western blotting (figure 5C). After 12 days of catecholaminergic differentiation, induced TH⁺ neuronal cells showed high TH expression, whereas TH was not detected in MSC (figure 5C). β-Actin expression was not affected during catecholaminergic differentiation (figure 5C).

Cell therapy with induced TH⁺ neuronal cells in experimental arthritis

To investigate the influence of in vitro-induced TH⁺ neuronal cells, these cells were adoptively transferred to animals with CIA. MSC- and PBS-treated mice were used as controls.

We studied tissue distribution of injected PKH26-labelled TH⁺ neuronal cells. After 24 h, induced TH⁺ neuronal cells were detected in paw joints, lymph nodes, spleen and adrenal gland (figure 6A). No labelled TH⁺ neuronal cells were found in brain, lung, heart, digestive organs or in kidneys.

CIA appeared on day 19–21 after immunisation, and score 10 was reached on day 22–25 after immunisation (figure 6B). Clinical arthritis scores of control mice showed a characteristic progression with strong increase between day 22 and 38 after immunisation and a maximum mean score of 25 score points on day 35–38 (figure 6B). In contrast, mice treated with 1 million induced TH⁺ neuronal cells developed a significantly milder arthritis (figure 6B, $p=0.025$ for comparison vs controls using general lineal model). Treatment effects of induced TH⁺ neuronal cells were observed from day 10 after adoptive transfer and a marked difference versus control animals remained until the end of observation (day 30 after one-shot cell treatment). MSC treatment also resulted in a milder but not significantly ameliorated arthritis (see online supplementary figure S3A, $p=0.222$ for comparison vs controls using general linear model).

The H&E staining of cryo-sections showed a higher number of cell nuclei in synovial tissue in phosphate-buffered saline (PBS) injected control paws. In contrast, in sections of MSC- or TH⁺ neuronal cell-treated paws cell infiltration was less pronounced (see online supplementary figure S4).

Since 6OHDA treatment induced a strong pro-inflammatory increase in late CIA,⁸ and since 6OHDA can deplete induced TH⁺ neuronal cells in vitro (figure 5B), we hypothesised that injection of 6OHDA increases arthritis severity in animals

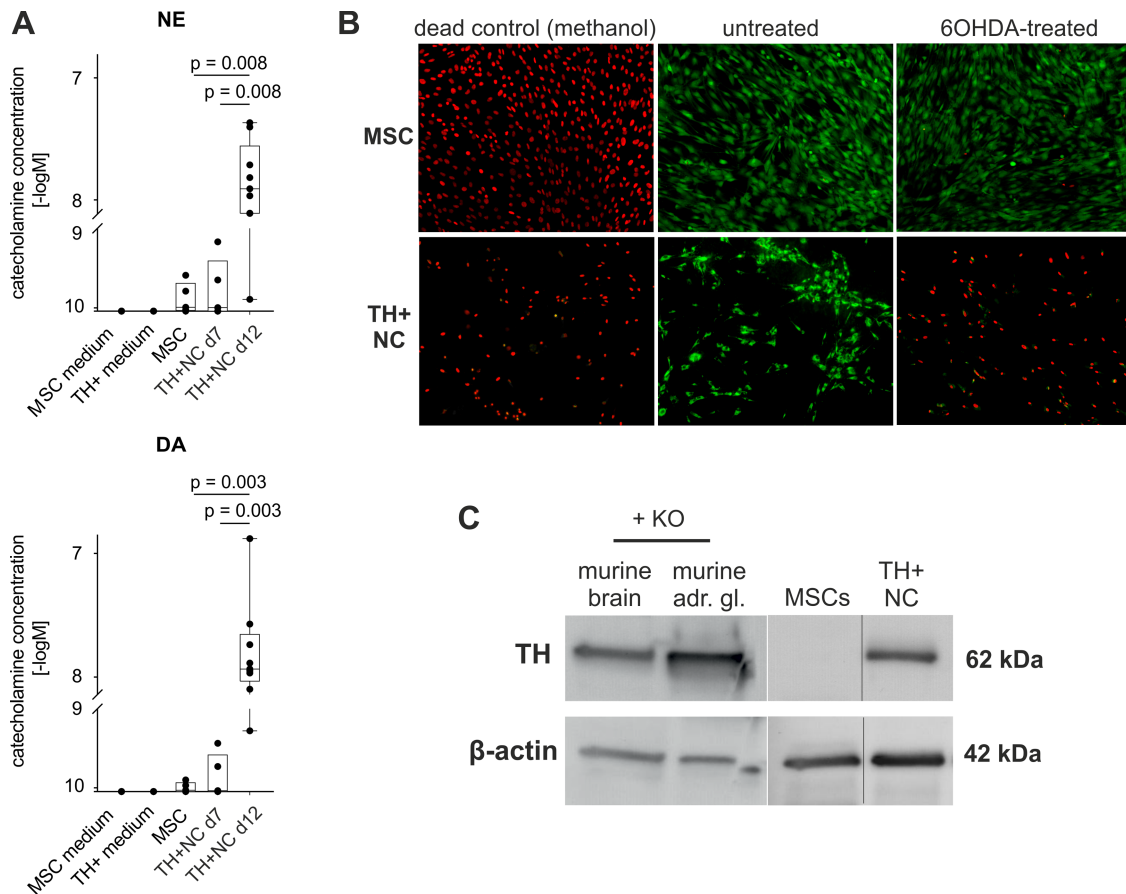


Figure 5 Catecholamine secretion and tyrosine hydroxylase (TH) expression in murine mesenchymal stem cell (mMSCs)-derived TH⁺ neuronal cells. (A) Secretion of catecholamines (NE, norepinephrine; DA, dopamine) in media without cells (mMSC medium, TH medium) and in supernatants of mMSCs and TH⁺ neuronal cells (n=8–10). Box plots are explained in legend to figure 1. (B) Targeted killing of TH⁺ neuronal cells by 6-hydroxydopamine (6OHDA) and detection of live/dead cells after 6OHDA treatment. (C) Western blot analysis of expression of TH in TH⁺ neuronal cells compared with mMSCs. TH⁺ NC, TH⁺ neuronal cells.

treated with TH⁺ neuronal cells. Indeed, intraperitoneal injection of 6OHDA 10 days after adoptive transfer resulted in a short initial fall followed by a steady increase of arthritis scores until day 30 after cell treatment (figure 6C). This reached the level of arthritic animals without cell treatment (figure 6C). No similar effects of 6OHDA were observed in control or MSC-treated animals (see online supplementary figure S3B, $p=0.966$ using general linear model; online supplementary figure S3C, $p=0.069$ using general linear model).

DISCUSSION

For three decades, anti-inflammatory effects of catecholamines such as norepinephrine are known when these neurotransmitters operate via β -adrenoceptors (reviewed in²⁷). Thus, the loss of sympathetic nerve fibres in inflamed tissue was interpreted as a pro-inflammatory event.^{2, 7, 28} Importantly, concomitant to the loss of sympathetic nerve fibres there appear newly formed cells that express TH, which we called TH⁺ cells or catecholaminergic cells,^{9, 10} but their role is unclear. While some authors hypothesised that these cells have pro-inflammatory activities,²⁹ TH⁺ cells might have anti-inflammatory capacities when neurotransmitter concentrations are high enough for β -adrenergic signalling.⁹

It was the starting point of the present work to find mechanisms that can increase TH protein expression and function in inflammatory cells in order to increase local catecholamine levels. An important factor to increase TH expression is

hypoxia.^{18, 19, 30, 31} In the present study, we demonstrated that TH expression increased when mixed synovial cells of OA and RA patients were incubated under hypoxic conditions. This confirms the work in other cell types.^{30, 31} Hypoxia reduced TNF levels in OA and RA synovial cells, downregulated IL-6/IL-8 in OA, but upregulated IL-6/IL-8 in RA. While several studies demonstrated differential results of hypoxia on various cytokines,^{32, 33} this is similarly ambiguous in primary synovial cells of RA and OA in our study (which are mainly macrophages and fibroblasts). At the moment, we have no conclusions why RA and OA cells behave in such a different way.

In order to demonstrate the specific hypoxia effect of TH on cytokine secretion, we blocked TH by the highly competitive antagonist α mpT. These hypoxia-mediated effects were enhanced when we used specific TH cofactors BH₄ and Fe²⁺, which are known to optimise TH activity. TNF inhibition in OA/RA synovial cells by hypoxia was abrogated by α mpT demonstrating specific effects via this catecholaminergic key enzyme. Similarly, hypoxia-induced effects on IL-6 secretion in OA/RA synovial cells were augmented with BH₄ and Fe²⁺ and, again, these changes were eliminated by α mpT. These experiments support hypoxia-induced effects on cytokines via an increase of TH.

Another important aspect in this research is determination of environmental catecholamines in the proximity of TH⁺ cells because high levels of norepinephrine are desired (β -adrenergic anti-inflammatory effects). In earlier studies, we demonstrated

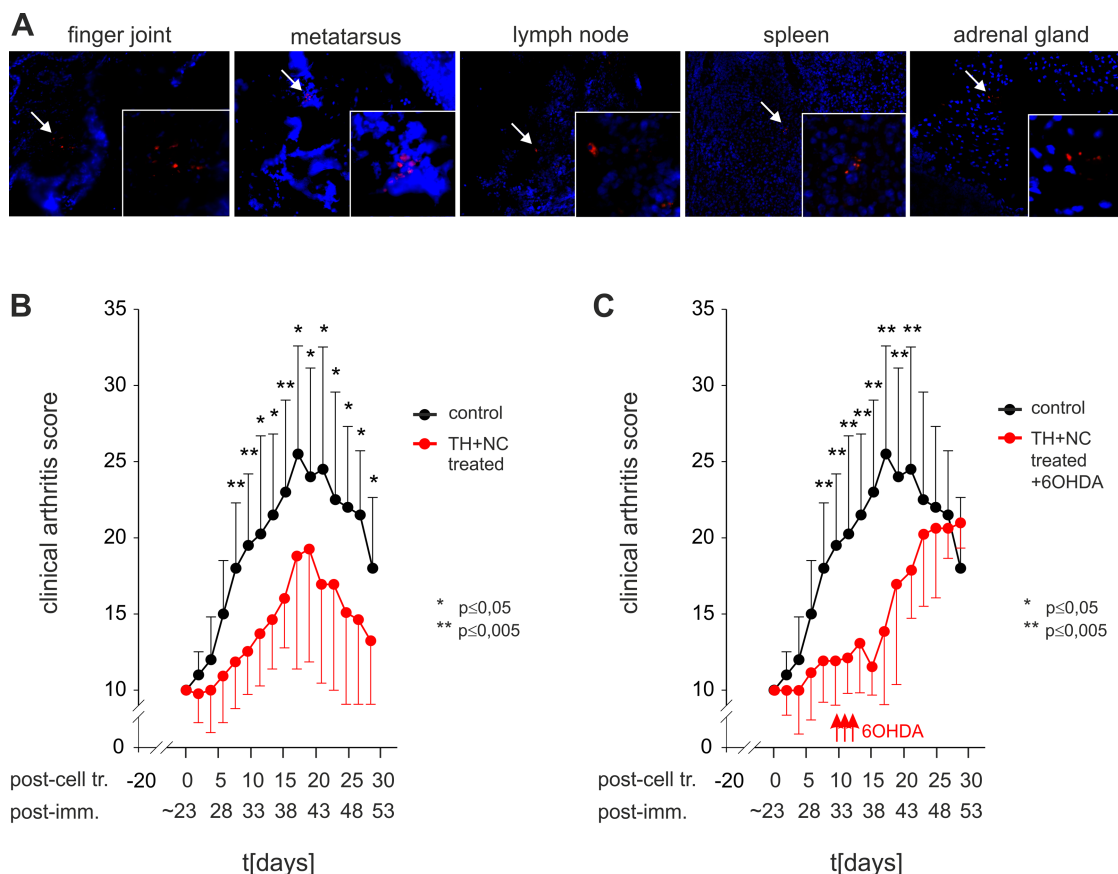


Figure 6 Generated tyrosine hydroxylase-positive (TH⁺) neuronal cells in experimental arthritis and organ distribution. (A) Detection of PKH26 prelabelled TH⁺ neuronal cells in paw joints, lymph nodes, spleen and adrenal gland of arthritic mice after intravenous injection. (B) Anti-inflammatory effects of TH⁺ neuronal cells on clinical score of experimental arthritis after intravenous injection compared with control arthritic mice (n=14). (C) Reversion of anti-inflammatory effects of TH⁺ neuronal cells by intraperitoneal treatment of arthritic mice with 6-hydroxydopamine (6OHDA) (n=14). post-cell tr, days after adoptive transfer of TH⁺ neuronal cells; post-immun, days after immunisation.

presence of catecholamines in cultures of synovial TH⁺ cells (5.9 pmol/L, 0.5 and 1.0 nmol/L)^{9–11} and in perfusion culture of synovial tissue (0.5–0.8 nmol/L).¹¹ In order to improve the detection of catecholamines, inhibitors of catecholamine degrading enzymes, MAO and COMT, were used (in addition to BH₄ and Fe²⁺ treatment). Under these conditions, norepinephrine levels rose to 10–100 nmol/L (10⁻⁷ mol/L). This indicates that environmental conditions can exist, which lead to high neurotransmitter levels relevant for β-adrenergic signalling. Importantly, by administration of MAO plus COMT blockers, inhibitory effects of hypoxia on TNF secretion in OA and RA cells was markedly increased, which indicates a specific catecholaminergic effect.

Results *in vitro* persuaded us to test effects of TH⁺ cells *in vivo* in experimental arthritis in mice. Since these cells cannot be separated by magnetic or fluorescent sorting techniques (no specific surface molecule), TH⁺ cells were generated using murine MSCs as described previously for human MSCs.²⁴ We called these cells TH⁺ neuronal cells.

Injected TH⁺ neuronal cells were found in paws but also in abdominal organs (lymph nodes, spleen, adrenal glands). The presence of TH⁺ neuronal cells in the joints 24 h after cell injection demonstrates clearly that TH⁺ neuronal cells survive *in vivo*, migrate and exert anti-inflammatory effects. The migration of induced TH⁺ neuronal cells into lymph nodes and spleen suggests an additional anti-inflammatory immune response contributing to possible favourable local effects.

Importantly, cellular therapy with TH⁺ neuronal cells led to a significant reduction of inflammation, which remained stable until the end of the observation period. One single injection of TH⁺ neuronal cells was sufficient to maintain anti-inflammatory effects for a long period of time. Similarly, MSC-treated mice developed milder arthritis confirming earlier studies.³⁴ However, MSCs act as key regulators of immune tolerance by inducing the generation/activation of Treg cells and not via the key enzyme of catecholamine production since these cells do not express TH. Positive effects of TH⁺ neuronal cells and MSC were confirmed by H&E staining.

In order to show that TH⁺ neuronal cells are responsible for milder arthritis, we depleted TH⁺ neuronal cells by treatment with 6OHDA as demonstrated earlier.¹⁰ During these experiments, a short decrease of arthritis score was observed during injection of 6OHDA. We hypothesise that this effect is most likely depending on acute catecholamine release from sympathetic nerve fibres and TH⁺ cells after 6OHDA injection, which is termed the initial 6OHDA norepinephrine burst. Such a catecholamine burst is well known after treatment with 6OHDA (fluffy coat, tachycardia, high activity). However, after this burst period, arthritis symptoms became significantly worse. These experiments confirm earlier experiments of our group in which injection of 6OHDA increased arthritis score in the late phase of CIA (approximately day 55).⁸ It is important to mention that control animals and MSC-treated animals receiving 6OHDA did not show worsening. In earlier experiments, we demonstrated

that 6OHDA ameliorates CIA when given before or until day 18 after immunisation, while CIA is aggravated when 6OHDA is given after day 45.^{8 35} However, administration of 6OHDA between day 18 and 45 had no effect (we called this interval transition phase).³⁵ Since we injected TH⁺ neuronal cells in this transition phase and since we observed worsening effects of 6OHDA in the transition phase, it is most likely that 6OHDA effects are specific through elimination of TH⁺ neuronal cells that were given in the transition phase.

In conclusion, this study demonstrated hypoxia-induced upregulation of TH. TH was responsible for inhibition of TNF in OA and RA synovial cells demonstrating an anti-inflammatory effect of increased TH function. Cellular therapy with TH⁺ neuronal cells demonstrated a clear anti-inflammatory influence of these cells in experimental arthritis. In future studies, we will target specific receptors (adrenergic and adenosinergic) in order to elucidate underlying molecular mechanisms of hypoxia driven TH-dependent anti-inflammatory phenomena in arthritis. This will help in developing novel cell therapy options for patients with RA.

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Contributors ZJ-L: discussing the concept, generation of data, generating draft figures, drafting parts of the paper, final approval. SC: generation of data, generating draft figures, revising the draft paper, final approval. FK, MF and TL: providing study tools and techniques, revising the draft paper, final approval. RHS: development of the concept, drafting parts of the paper, generating final figures, final approval.

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Competing interests None.

Ethics approval Ethics Committee of the University of Regensburg.

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