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Epicutaneous Immunization with Collagen Induces TCRαβ Suppressor T Cells That Inhibit Collagen-Induced Arthritis

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Key Words
Epicutaneous immunization · TCRαβ suppressor T cells · Collagen-induced arthritis

Abstract
Background: We have shown previously, in an animal model of multiple sclerosis and in TNBS-induced colitis, that epicutaneous (EC) immunization with protein antigen induces T suppressor cells that strongly inhibit the inflammatory response in contact hypersensitivity reactions. ***Methods:*** EC immunization was performed by applying to the shaved skin of the mouse dorsum a gauze patch soaked with a solution containing various amounts of type II collagen (COLL II) in a volume of 100 µl of PBS on days 0 and 4. On day 7 the patches were removed and mice were intradermally (i.d.) immunized with COLL II to induce collagen-induced arthritis (CIA). ***Results:*** Our study shows that EC immunization with 100 or 30 µg of COLL II reduces disease severity, whereas lower doses (10 or 3 µg) do not affect CIA. Decreased disease severity observed after EC immunization with COLL II correlates with reduced myeloperoxidase activity in joint tissue and with reduced production of anti-citrullinated protein and anti-COLL II IgG2a antibodies. Transfer experiments show that EC immunization with COLL II induces suppressor cells that belong to the population of TCRαβ lymphocytes and that EC-induced suppression declines with time. Both in vitro and in vivo experiments show that IL-17A plays an important role in EC-induced suppression of CIA. EC application of COLL II at the first signs of CIA also results in disease suppression. ***Conclusions:*** The suppression of inflammatory responses by T suppressor cells induced through EC immunization of a protein antigen may become an attractive noninvasive therapeutic method for a variety of clinical situations.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects about 1% of the adult population and occurs twice as frequently in women as in men [1]. The onset may appear at any age, but the peak incidence comes in the 25- to 55-year age range [2]. Because the disease affects persons in the economically productive age range, it presents an ever increasing economic and social burden on the quality of life of patients and on their ability to work.

RA is an autoimmune disease that is characterized by chronic inflammation in the joints and by synovial hyperplasia, leading to cartilage and bone loss. The pathogenesis of RA appears to be based on inflammatory responses mediated by self-reactive $CD4^+$ Th1 and Th17 lymphocytes [3–5].

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Animal studies are a valuable source of information on the role of the immune system in RA pathogenesis. The most common animal model of RA is collagen-induced arthritis (CIA) that can be induced in genetically prone strains of mice, rats, rabbits or rhesus monkeys [6].

Various drugs are used in the therapy of RA, commonly including nonsteroidal anti-inflammatory drugs. They principally relieve the symptoms of RA with minimal impact on the disease process through their anti-inflammatory and analgesic activities, but have significant adverse side effects [7]. Further treatment of RA includes disease-modifying antirheumatic drugs (DMARDs) such as sulfasalazine, methotrexate and hydroxychloroquine. These drugs also cause a number of severe side effects [8]. In recent years, RA therapy has employed biologic DMARDs that target cytokines or their receptors, or are directed against other cell surface molecules [9]. These drugs interfere with the immune mechanisms underlying RA pathology. Biologic DMARDs face barriers such as limited availability, a high price and induction of nonspecific immunosuppression [10].

Therefore, there is a great need to develop new therapies that are noninvasive, are free of side effects and specifically eliminate the inflammatory reaction underlying RA. Several efforts have been made to develop a 'vaccine' which would prevent the onset of autoimmune diseases, including RA. One of these methods relies on the induction of oral tolerance. In the animal model it has been shown that feeding with collagen induces strong tolerance in the periphery and by this mechanism significantly reduces symptoms of CIA [11]. Similar observations have also been made in experimental autoimmune encephalomyelitis (EAE), which is an example of another autoimmune disease mediated by Th1/Th17 lymphocytes. However, clinical trials have shown no benefit in RA or multiple sclerosis patients fed with collagen or myelin basic protein, respectively [12, 13].

Our previous work both in contact hypersensitivity (CHS) [14, 15], EAE [16, 17] and TNBS (trinitrobenzene sulfonic acid)-induced colitis [18] has demonstrated that EC immunization with protein antigen prior to the induction of T-mediated immune response causes strong suppression of the response. Our further work employing allogeneic skin grafts has shown that EC immunization with a protein antigen delays graft rejection [19]. Moreover, we have recently reported for the first time that EC immunization with myelin peptides generates tolerogenic responses and attenuates autoimmunity in patients with multiple sclerosis [20, 21].

In the current work we show that EC immunization with protein antigen protects against CIA and that this protection is transferable with TCRαβ T suppressor (Ts) cells. Both in vitro and in vivo experiments show that IL-17A is involved in the negative regulation of inflammatory responses during CIA.

Materials and Methods

Mice

Male DBA/1 mice, 8–12 weeks old, were obtained from the breeding unit of the Department of Medical Biology, Jagiellonian University School of Medicine. Mice were fed autoclaved food and water. All experiments were conducted according to the guidelines of the Animal Use and Care Committee of the Jagiellonian University School of Medicine.

Reagents

Bovine type II collagen (COLL II), complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were obtained from Chondrex Inc. (Redmond, Wash., USA). Hexadecyltrimethylammonium bromide, o-dianisidine dihydrochloride, hydrogen peroxide and LPS (from *Escherichia coli* 026:B6) were purchased from Sigma (St. Louis, Mo., USA). RPMI 1640 and fetal calf serum were from Life Technologies (Grand Island, N.Y., USA). Low-Tox rabbit complement (RC) was from Pel-Freeze Biologicals (Brown Deer, Wisc., USA).

Monoclonal Antibodies and Hybridoma

The following purified anti-mouse mAbs were used: anti-TCRβ (clone H57-597) from Dr. R. Kubo of Cytel Inc. (La Jolla, Calif., USA) and anti-TCRδ (clone UC7-13D5) from Dr. J. Bluestone (University of California, San Francisco, Calif., USA). Purified rat anti-mouse IL-17, rat anti-mouse TGF-β1 and biotinylated anti-mouse, anti-human, anti-pig TGF-β1 antibodies were obtained from BD Biosciences (San Diego, Calif., USA). The isotype control antibody (rat IgG) was from Sigma (St. Louis, Mo., USA).

EC Immunization with Protein Antigen

EC immunization (fig. 1a) was performed by applying to the shaved skin of the mouse dorsum a 1-cm^2 gauze patch soaked with a solution containing graded amounts (3–100 μg/mouse) of COLL II in a volume of 100 μl of PBS on day 0. The patch was secured by adhesive tape wrapped around the midsection. Positive control mice were patched with PBS alone. The patch was left in place from day 0 until day 4, when it was replaced with a fresh patch. On day 7 the mice were intradermally (i.d.) immunized with COLL II in CFA as described below. In some experiments lymph organs isolated from donors EC immunized with COLL II were used as a source of suppressor cells.

Induction and Gross Assessment of CIA

Immunization and arthritis evaluation were performed as described by Kang et al. [22] with some modification. DBA/1 mice were injected i.d. at the base of the tail with 100 μg of COLL II emulsified in CFA on day 0 and boosted with 100 μg of COLL II in IFA on day 21 after the first immunization. To accelerate the development of arthritis, 40 μg of LPS in sterile PBS was intraperitoneally (i.p.) injected on day 28. Animals were observed daily for the presence of arthritis and the clinical severity of disease was

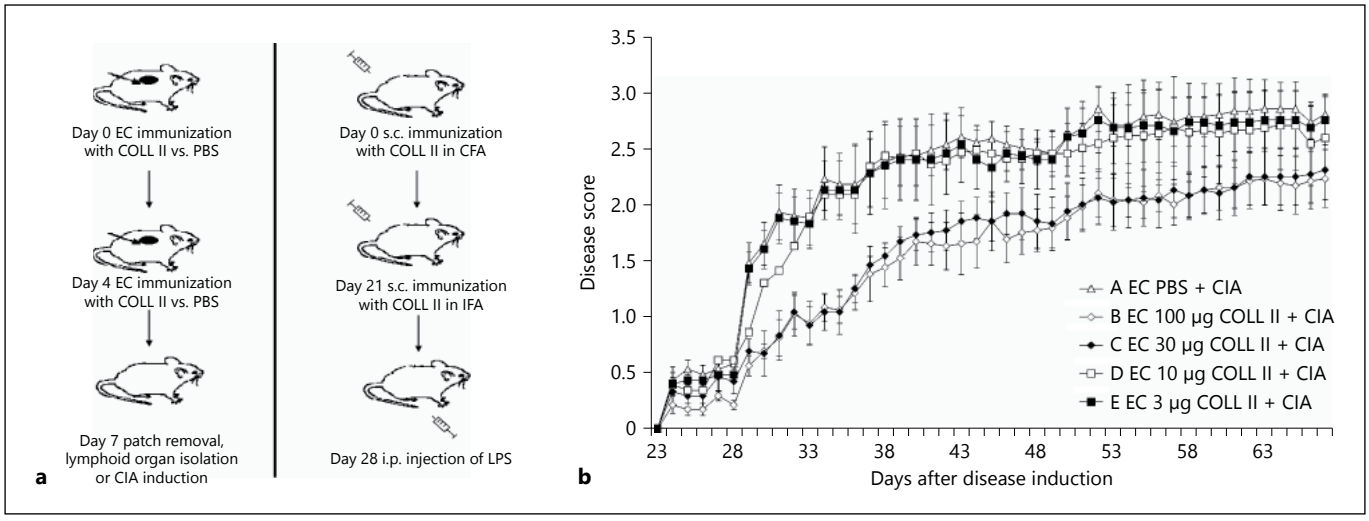

Fig. 1. EC application of COLL II before CIA induction decreases the severity of disease. **a** Mice were EC immunized with COLL II or treated with PBS on days 0 and 4. Then, patches were removed on day 7 and CIA was induced. To this end mice were i.d. immunized with COLL II in CFA (day 0) and boosted with COLL II in IFA on day 21 after the first immunization. To accelerate the development of arthritis, 40 µg of LPS in sterile PBS was i.p. injected on day 28. **b** Mice were patched with different doses of COLL II (group B – 100 µg; group C – 30 µg; group D – 10 µg; group E – 3 µg) or PBS (group A) prior to CIA induction. Each group contained 6–8 mice. Data are presented as the mean arthritis score in each group ± SE. Statistical significance between groups emerged after day 28: $p < 0.05$ for groups B and C vs. group A.

scored for each paw on a scale of 0–4 [22, 23]. The criteria for the grading were as follows: 0 – no evidence of erythema and swelling; 1 – mild erythema and swelling of the wrist or the ankle; 2 – moderate erythema and swelling from the wrist to the metacarpal joints or from the ankle to the metatarsal joints; 3 – severe erythema and swelling of the entire paw, including digits; 4 – maximal erythema and swelling of the paw.

Adoptive Transfer of EC-Induced Suppression

Mice were EC immunized with 100 µg/mouse of COLL II for 1 week. On day 7 axillary and inguinal lymph nodes, spleens, thymus or mesenteric lymph nodes were collected and single cell suspensions were prepared under aseptic, conditions. Then, 2.5×10^7 lymphoid cells were transferred i.v. into naïve syngeneic recipients that immediately underwent arthritis induction. To determine the minimal number of EC-induced suppressor cells that could inhibit CIA, graded numbers (4×10^5 to 2.5×10^7) of axillary and inguinal lymph node cells (ALNC) were injected i.v. into naïve recipient mice that immediately underwent arthritis induction. Animals were scored for arthritis as described above.

Phenotype of Suppressor Cells

To determine the phenotype of EC-induced suppressor cells in vivo, ALNC isolated from mice EC treated with COLL II were incubated in PBS on ice with purified anti-TCRβ or anti-TCRδ mAbs (1 µg Ab/10^6 cells), or with PBS alone for 45 min. The cells were washed and incubated with a predetermined dilution of RC for 60 min at 37°C, and then washed and resuspended in PBS. Next, cells were counted and cell viability was assessed by trypan blue exclusion. A suppression control consisting of 2.5×10^7 EC-induced suppressor cells treated with RC alone, or 2.5×10^7 cell aliquots of EC-induced suppressor cells treated with appropriate mAbs and RC were transferred i.v. into naïve recipients that underwent arthritis induction. Mice were observed daily and scored for arthritis as described above.

Myeloperoxidase Assay

Neutrophil infiltration to the inflamed joints was indirectly quantitated using a myeloperoxidase (MPO) assay, as described previously [24]. Joint tissues were prepared by first removing the skin and separating the limb below the ankle joint. Joint tissues were homogenized in 0.5% hexadecyltrimethylammonium bromide, pH 6.0 (50 mg of tissue/ml). The homogenates were freeze-thawed 3 times and centrifuged at 40,000 *g*. Then, 0.1-ml aliquots were mixed with 2.9 ml of phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride and 5×10^{-4} % H_2O_2, and incubated at 25°C for 20 min. The absorbance was measured at 460 nm in 96-well flat-bottom plates. MPO activity was expressed in units per protein concentration (U/g of protein).

Measurement of Serum Anti-COLL II Antibody Levels

DBA/1 mice were EC exposed to PBS or COLL II for 1 week prior to the induction of CIA. Animals were monitored for arthritis, serum samples were collected on day 55 postimmunization and the level of COLL II-specific IgG1 and IgG2a antibodies was measured with the use of mouse anti-COLL II IgG1 and IgG2a subtype assay kits from Chondrex Inc.

Measurement of Serum Anti-Cyclic Citrullinated Peptide Antibodies

Anti-cyclic citrullinated peptide (CCP) antibodies were detected using a commercially available second-generation enzyme-linked immunosorbent assay kit (ImmunoscanRA Anti-CCP Test kit,

Euro-Diagnostica, Malmö, Sweden), and the secondary antibody was substituted with rabbit anti-mouse Ig, HRP-conjugated from DAKO (Glostrup, Denmark). Anti-CCP antibodies were measured on mouse serum collected from mice with induced CIA, or from mice that were EC tolerized with COLL II before CIA induction. The samples were diluted 10 times with dilution buffer (provided by the manufacturer) before the test. Samples were measured at 450 nm.

Measurement of Cytokines in Culture Supernatants

To determine the influence of EC immunization on cytokine production, DBA/1 mice were EC exposed to PBS or COLL II as described in Material and Methods. Then, on day 7, ALNC were collected and processed under aseptic conditions. Additionally, to test if EC immunization with COLL II affects cytokine production in mice with induced arthritis, DBA/1 mice were EC exposed to PBS or COLL II prior to CIA induction. Then, on day 10 after i.d. injection of COLL II with CFA, mice were sacrificed and ALNC were collected. ALNC (3×10^6) from mice EC treated with PBS (control group) or COLL II were cultured in 1 ml of RPMI 1640 medium supplemented with 5% fetal calf serum in the presence of 100 μg/ml of COLL II. Cells were distributed in triplicate wells in flat 24-well Falcon plates. After 48 h in culture, supernatants were collected and then tested for their cytokine concentration [25]. Culture supernatants were tested for IL-4, IL-6, IL-10, IL-12p70, TGF-β, IFN-γ and TNF-α concentration with the use of BD OptEIA Set from BD Biosciences. The concentration of IL-8 was measured with ELISA kit from MyBioSource (San Diego, Calif., USA). The concentration of IL-17E was evaluated with an ELISA kit (R&D Systems Inc., Minneapolis, Minn., USA). The concentration of IL-17A was measured with ELISA kit from eBioscience Inc. (San Diego, Calif., USA). The concentration of IL-35 was tested with the use of ELISA kit from Biolegend (San Diego, Calif., USA). For estimating TGF-β concentrations, supernatants were first acidified with 1 N HCl diluted 1:25 and then neutralized with 1 N NaOH to 7.0 pH in the same proportion. Cytokine concentrations in culture supernatants were measured by sandwich ELISA using monoclonal rat anti-mouse TGF-β antibodies as the capture antibody and biotinylated monoclonal rat anti-mouse, anti-human and anti-pig TGF-β antibodies as the secondary antibody. The reaction was developed with horseradish peroxidase streptavidin, followed by o-phenylenediamine and H_2O_2 as the substrate, and was stopped with 1 N H_2SO_4. The optical density of each well was measured in a 96-well plate reader at 492 nm. All determinations were done in triplicate. A standard curve was generated with recombinant mouse TGF-β. The lower concentration limit was 30 pg/ml TGF-β.

Staining for Transcription Factor RORγt

ALNC from mice EC treated with PBS or COLL II were first incubated with anti-CD16/32 mAb (2.4G2; BD Pharmingen) and then stained with anti-TCRβ-APC-Cy7 mAb (BD Biosciences). Samples were then intracellulary stained with anti-RORγt-PE mAb (BD Bioscience) using a mouse fixation and permeabilization kit (eBioscience, San Diego, Calif., USA). Stained samples were then assessed by flow cytometry using a FACS Canto II (BD Biosciences) and data was analyzed using FACSDiva software.

Statistical Analysis

Data in graphs are shown as the mean ± SE. Statistical significance was estimated by Mann-Whitney test, Kruskal-Wallis test and unpaired t test. Statistical significance was set at $p < 0.05$.

Results

EC Immunization with COLL II Prior to Induction of Collagen-Induced Arthritis Alleviates Disease

DBA/1 mice were EC immunized with graded doses of COLL II (100, 30, 10 or 3 μg/animal) or exposed to PBS alone as described in Materials and Methods (fig. 1a), and the patches were removed on day 7. Mice were i.d. immunized with COLL II and CFA on day 0, then boosted with COLL II in IFA on day 21, and finally i.p. treated with LPS. Animals were scored from day 21 after immunization with collagen and CFA. EC immunization with COLL II prior to the induction of CIA reduced disease severity in a dose-dependent manner (fig. 1b). Mice exposed to doses of COLL II 100 and 30 μg/animal prior to induction of CIA developed significantly less severe disease when compared to positive control (mice EC treated with PBS before disease induction; groups B and C vs. group A). EC immunization with lower doses of COLL II, such as 10 and 3 μg/animal, did not affect disease severity when compared to positive control (groups D and E vs. group A). These data strongly suggest that EC immunization with COLL II prior to CIA induction significantly reduces disease severity and that the strength of suppression is dependent on the tolerizing dose of the antigen applied to the skin.

Decreased Disease Severity Observed after EC Immunization with COLL II Correlates with Reduced MPO Activity in Joint Tissue

As the optimal dose of EC-applied COLL II for amelioration of disease ranged between 30 and 100 μg/mouse, we decided to use the dose of 100 μg of COLL II per animal in further experiments. Mice EC immunized with 100 μg of COLL II developed less severe disease when compared to positive control (group B vs. group A; fig. 2a). Decreased disease severity correlated with reduced MPO activity in joint tissue tested on days 33, 48 and 63 after CIA induction (group B vs. group A; fig. 2b).

EC Immunization with COLL II before CIA Induction Results in Decreased Production of Anti-COLL II and Anti-CCP Antibodies

The involvement of anti-COLL II IgG2a antibody in the pathogenesis of CIA has been shown previously [5, 26]. Thus, we decided to determine the influence of EC immunization on the production of anti-COLL II IgG1 and IgG2a antibodies. To this end, mice were EC treated with PBS or COLL II prior to CIA induction. On day 55 after CIA induction the animals were bled and sera were used for antibody estimation.

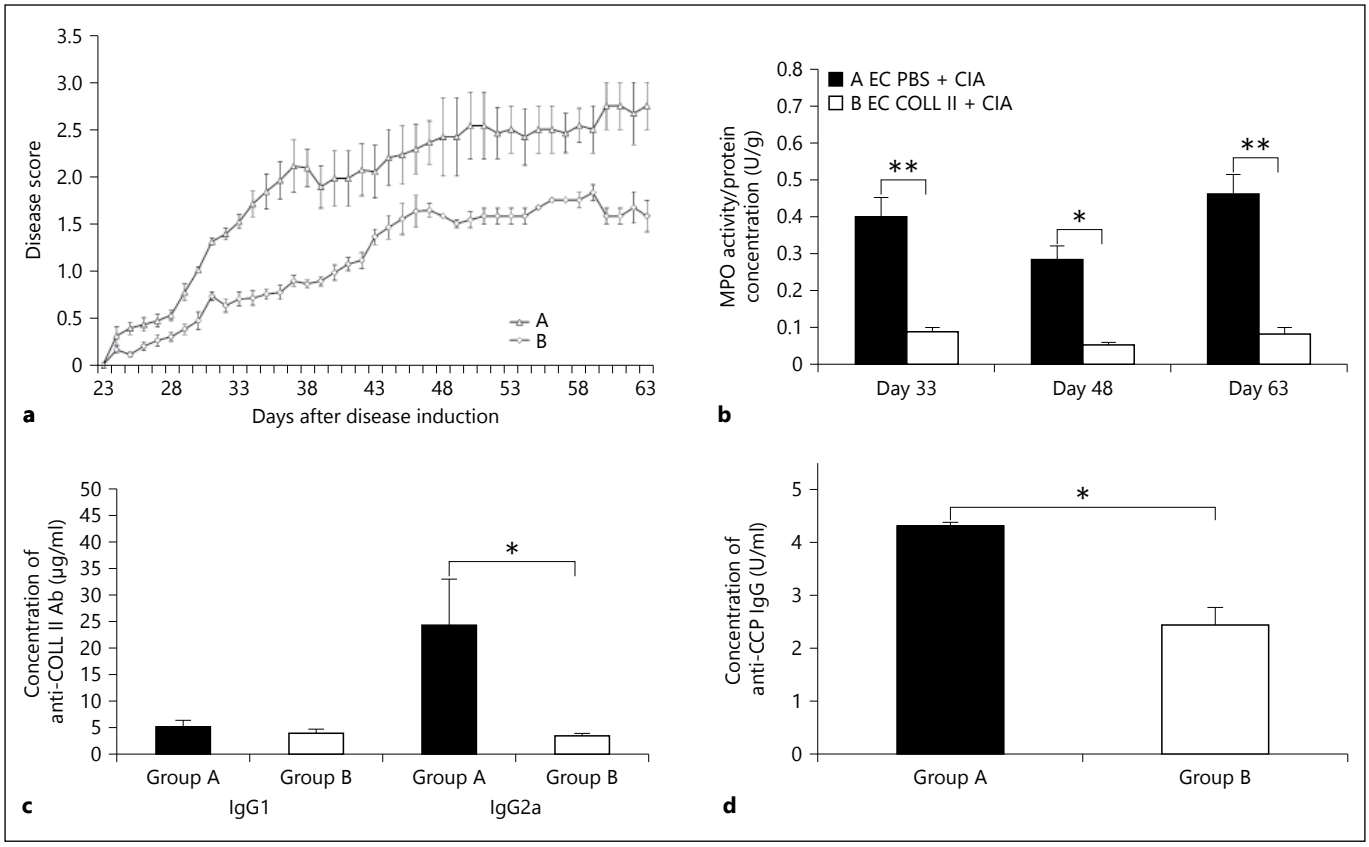

Fig. 2. EC application of COLL II before CIA induction inhibits MPO activity and production of anti-COLL II and anti-CCP antibodies. **a** COLL II (100 µg/mouse; group B) or PBS (group A) were applied epicutaneously prior to CIA induction. Each group contained 11 mice. Data are presented as the mean arthritis score in each group ± SE. Statistical significance between groups emerged after day 28: $p < 0.05$ for group B versus group A. **b** The level of MPO activity was evaluated in the joint tissue isolated on days 33, 48 and 63 after disease induction. Each group contained 5–8 samples. Values are presented as the mean ± SE. * $p < 0.05$; ** $p < 0.01$. **c** The level of anti-COLL II IgG1 and IgG2a antibodies was measured on day 55 after CIA induction. Each group contained 6 samples. Values are presented as the mean ± SE. * $p < 0.05$. **d** The level of anti-CCP antibodies was evaluated on day 55 after CIA induction. Each group contained 6 samples. Values are presented as the mean ± SE. * $p < 0.05$.

EC immunization with COLL II before CIA induction significantly decreased production of anti-COLL II IgG2a antibodies but did not affect the production of anti-COLL II IgG1 antibodies when compared with mice EC treated with PBS alone (fig. 2c). Treatment with either COLL II or PBS without subsequent CIA induction did not affect the production of anti-COLL II collagen antibodies (data not shown).

It has been shown previously that antibodies against citrullinated proteins are centrally involved in the pathogenesis of autoimmune arthritis [27]. Thus, we decided to test whether observed protection from disease after EC immunization with COLL II correlates with the production of anti-CCP antibodies. EC immunization with COLL II prior to CIA induction resulted in a decreased production of anti-CCP antibodies when compared to positive control (group B vs. group A; fig. 2d). Antibodies against CCP were not found in mice EC treated with COLL II or PBS without CIA induction (data not shown).

Skin-Induced Tolerance Can Be Transferred into Naïve Recipients

We sought to determine whether skin-induced tolerance can be transferred with cells of the immune system. Thus, 2.5×10^7 ALNC (group B), spleen cells (SPLC; group C), thymocytes (Thy; group D) or mesenteric lymph node cells (MLNC; group E) were isolated from mice EC immunized with COLL II for 1 week. These cells were transferred into naïve recipients that were then immunized to induce CIA and scored for arthritis. Positive control con-

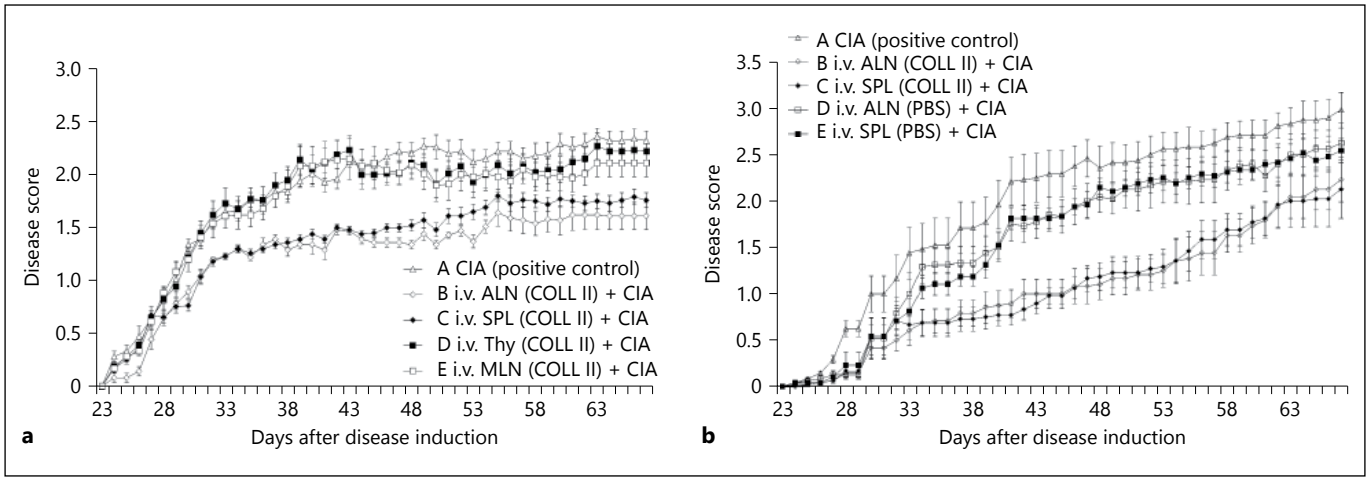

Fig. 3. a Skin-induced tolerance can be transferred into naïve recipients. 2.5×10^7 ALNC (group B), SPLC (group C), Thy (group D) or MLNC (group E) from mice patched with COLL II were transferred i.v. into naïve recipients that underwent CIA induction. The positive control (group A) consisted of mice that did not receive any cell transfer before disease induction. **b** 2.5×10^7 ALNC (group B) or SPLC (group C) from mice patched with COLL II or 2.5×10^7 ALNC (group D) or SPLC (group E) from PBS-patched donors were transferred i.v. into naïve recipients prior to CIA induction. Each group contained 9 mice. Data are presented as the mean arthritis score in each group ± SE. Statistical significance between groups emerged after day 28: $p < 0.001$ for groups B and C vs. group A.

sisted of mice that did not receive any cell transfer before disease induction. ALNC and SPLC from tolerized donors were able to suppress CIA in actively immunized mice when compared to positive control (groups B and C vs. group A; fig. 3a). On the other hand, transfer of MLNC and Thy prior to CIA induction did not affect disease severity when compared to positive control (groups D and E vs. group A; fig. 3a). Additionally, the data presented in figure 3b ruled out nonspecific suppressor activity of transferred cells, as adoptive transfer of 2.5×10^7 ALNC (group D) or SPLC (group E) isolated from PBS-patched donors did not significantly affect disease severity.

Activity of Skin-Induced Suppressor Cells Is Dose-Dependent

We attempted to find the minimal number of skin-induced suppressor cells that were able to inhibit the development of CIA. Thus, graded numbers (4×10^5 to 2.5×10^7) of ALNC (groups B–E, respectively) were transferred i.v. into naïve recipient mice that immediately underwent arthritis induction. Animals that did not receive any cell transfer before CIA induction functioned as a positive control (group A). Suppressor activity of ALNC isolated from COLL II patched mice was dose dependent (fig. 4), and even the lowest dose of used suppressor cells (4×10^5 of ALNC/animal) significantly alleviated disease (group B vs. group A).

EC Immunization with COLL II Induces TCRαβ$^+$ Suppressor Cells That Reduce the Severity of CIA

In order to determine the in vivo phenotype of the suppressor cells, ALNC from mice EC immunized with COLL II were treated with either anti-TCRβ or anti-TCRδ mAb and RC, or with RC alone (suppression control). These cells were transferred to naïve syngeneic recipients that were i.d. immunized with COLL II in CFA to induce CIA. Positive control animals did not receive any cells before the induction of disease. Skin-induced suppressor cells that protect from CIA belong to the population of TCRαβ$^+$ lymphocytes, whereas TCRγδ$^+$ cells do not seem to be involved in EC-induced suppression (groups C and D vs. group B; fig. 5).

Skin-Induced Suppression Declines with Time

To determine the duration of skin-induced suppression, donor mice of the suppressor cells were EC immunized with COLL II 1, 2, 4 or 6 weeks before their use. The suppressor cells induced at different time points (groups B–E, respectively; fig. 6) were transferred i.v. into naïve recipient mice that immediately underwent arthritis induction. Positive control animals received no cell transfer before CIA induction (group A). Skin-induced suppression declined with time (groups B–E vs. Group A; fig. 6). Maximal protection from CIA was observed when the suppressor cells were used 1 or 2

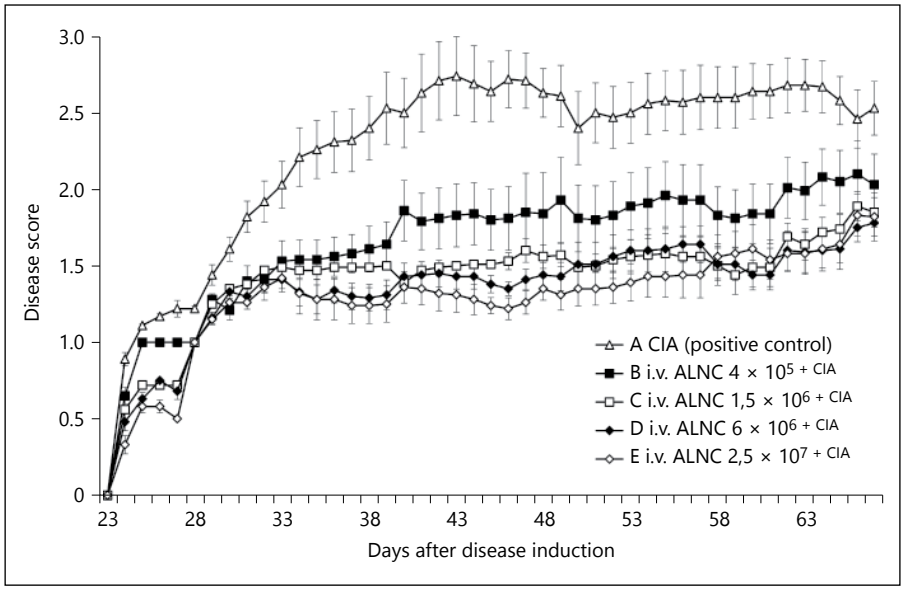

Fig. 4. Dose response activity of the skin-induced suppressor cells. Graded numbers of ALNC (4×10^5 – group B; 1.5×10^6 – group C; 6×10^6 – group D; 2.5×10^7 – group E) were transferred i.v. into naïve recipient mice prior to CIA induction. The positive control contained animals that did not receive any cell transfer before CIA induction (group A). Each group contained 9–10 mice. Data are presented as the mean arthritis score in each group ± SE. Statistical significance between groups emerged after day 28: $p < 0.05$ for group B versus group A; $p < 0.01$ for groups C and D versus group A; $p < 0.001$ for group E versus group A.

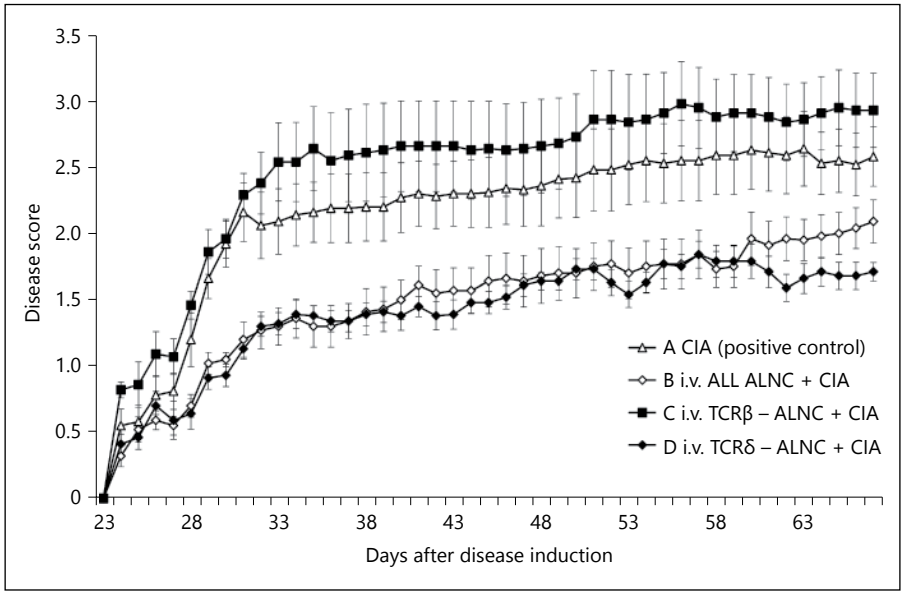

Fig. 5. TCRαβ$^+$ suppressor cells induced by EC immunization with COLL II reduce the severity of CIA. ALNC from mice EC immunized with COLL II were treated with RC alone (group B) or anti-TCRβ (group C) or anti-TCRδ mAb (group D) and RC. Then, 2.5×10^7 of EC-induced suppressor cells treated with RC alone (suppression control), or 2.5×10^7 cell aliquots of EC-induced suppressor cells treated with appropriate mAb and RC were transferred i.v. into naïve recipients that underwent CIA induction. Positive control animals did not receive any cells before the induction of disease (group A). Each group contained 7–8 mice. Data are presented as the mean arthritis score in each group ± SE. Statistical significance between groups emerged after day 28: $p < 0.05$ for groups B and D versus group A.

weeks after their induction (groups B and C), but 4-week suppressor cells were still able to alleviate disease (group D).

EC Exposure to COLL II Induces Production of IL-17A by ALNC

To determine if cytokines are involved in skin-induced tolerance, we measured the concentration of the proinflammatory cytokines TNF-α, IL-6, IL-8, IL-12, IL-17A and IFN-γ. Additionally, in this experiment, we tested if EC immunization with COLL II affects the synthesis of the anti-inflammatory cytokines IL-4, IL-10, TGF-β, IL-17E and IL-35. EC immunization with COLL II (group B) did not affect production of IL-4, IL-6, IL-8, IL-10, IL-12, IL-17E, IL-35, TGF-β and IFN-γ when compared to PBS-treated mice (group A; fig. 7a). There was a significant difference in IL-17A production between COLL II (group B) versus PBS-treated (group A) animals. Additionally, EC immunization with COLL II slightly but significantly increased TNF-α production when compared

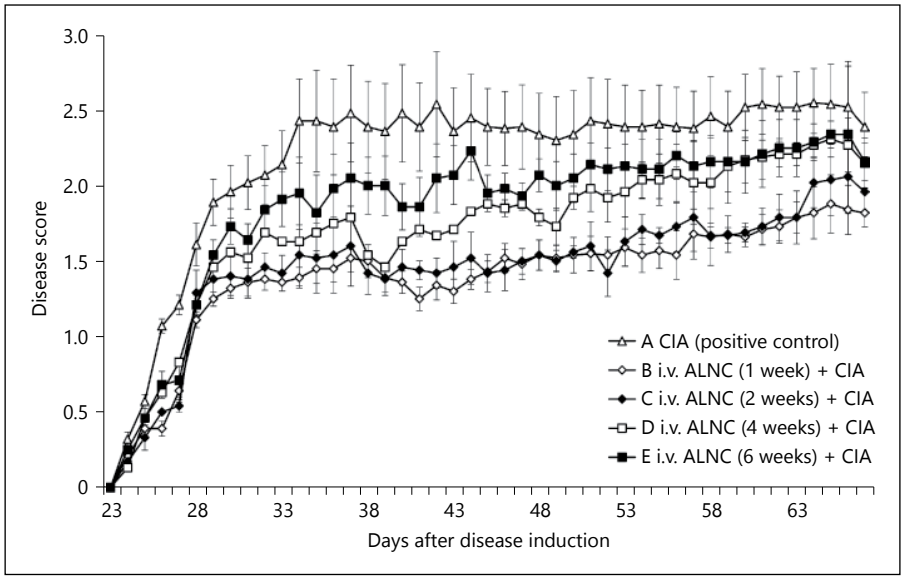

Fig. 6. Skin-induced suppression declines with time. Naïve recipient mice were injected i.v. with suppressor cells (2×10^7/mouse) induced in donors by EC COLL II immunization 1 (group B), 2 (group C), 4 (group D) or 6 weeks (group E) before CIA induction. The control group did not receive any cell transfer before CIA induction (group A). Each group contained 6–7 mice. Data are presented as the mean arthritis score in each group ± SE. Statistical significance between groups emerged after day 28: $p < 0.05$ for groups B and C versus group A.

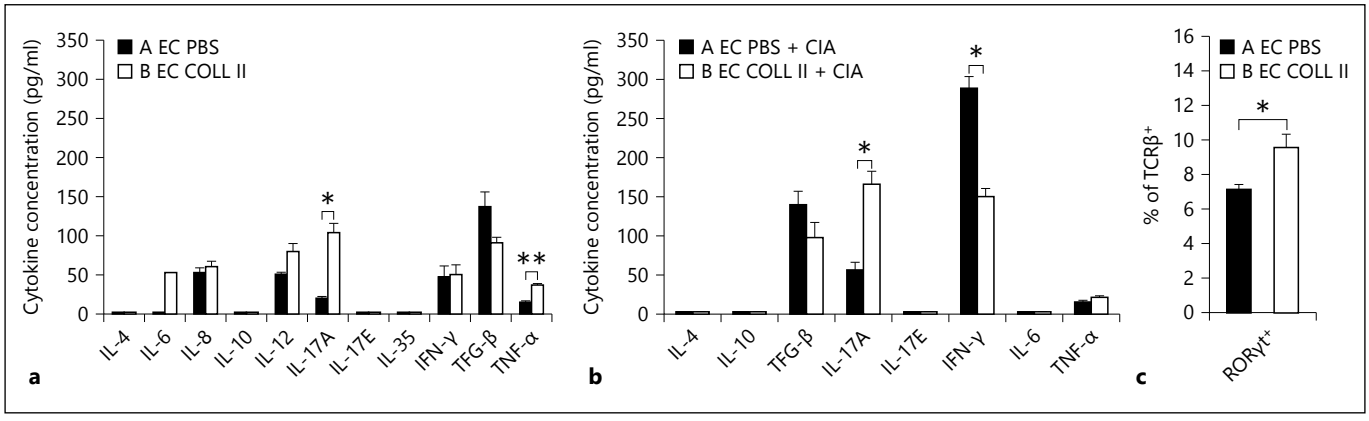

Fig. 7. EC exposure to COLL II induces production of IL-17A and inhibits production of IFN-γ by lymph node cells. **a** DBA/1 mice were EC exposed to PBS (group A) or COLL II (group B). On day 7 ALNC were collected and cultured in the presence of 100 μg/ml of COLL II for 48 h. The level of IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IL-17E, IL-35, IFN-γ, TGF-β and TNF-α was measured by ELISA. Each group contained 4 samples. Values are presented as the mean ± SE. * $p < 0.05$; ** $p < 0.01$. **b** DBA/1 mice were EC exposed to PBS or COLL II prior to CIA induction (groups A and B, respectively). On day 10 after i.d. injection of COLL II with CFA, ALNC were cultured in the presence of 100 μg/ml of COLL II for 48 h. The level of IL-4, IL-10, TGF-β, IL-17A, IL-17E, IFN-γ, IL-6 and TNF-α was measured by ELISA. Each group contained 4 samples. Values are presented as the mean ± SE. * $p < 0.05$. **c** DBA/1 mice were EC treated with PBS (group A) or COLL II (group B). On day 7 ALNC were isolated and stained with anti-TCRβ-APC-Cy7 mAb. After fixation and permeabilization ALNC were incubated with anti-ROR-γt-PE mAb and analyzed by flow cytometry. Each group contained 4 samples. ROR-γt expression is presented as the mean ± SE. * $p < 0.05$.

to PBS-treated mice (group B vs. A). Additionally, flow cytometry analysis showed that EC immunization with COLL II increases the percentage of TCRαβ$^+$ cells expressing RORγt when compared to PBS-treated mice (group B vs. group A; fig. 7c). There was no difference in the percentage of TCRαβ$^+$ CD4$^+$ FoxP3$^+$ Treg cells between tested groups (data not shown).

EC Immunization with COLL II Prior to Induction of CIA Inhibits the Production of IFN-γ

To test if EC immunization with COLL II affects cytokine production in mice with CIA, DBA/1 mice were EC exposed to PBS or COLL II prior to CIA induction. Then, on day 10 after i.d. injection of COLL II plus CFA, mice were sacrificed and ALNC were collected and cultured in

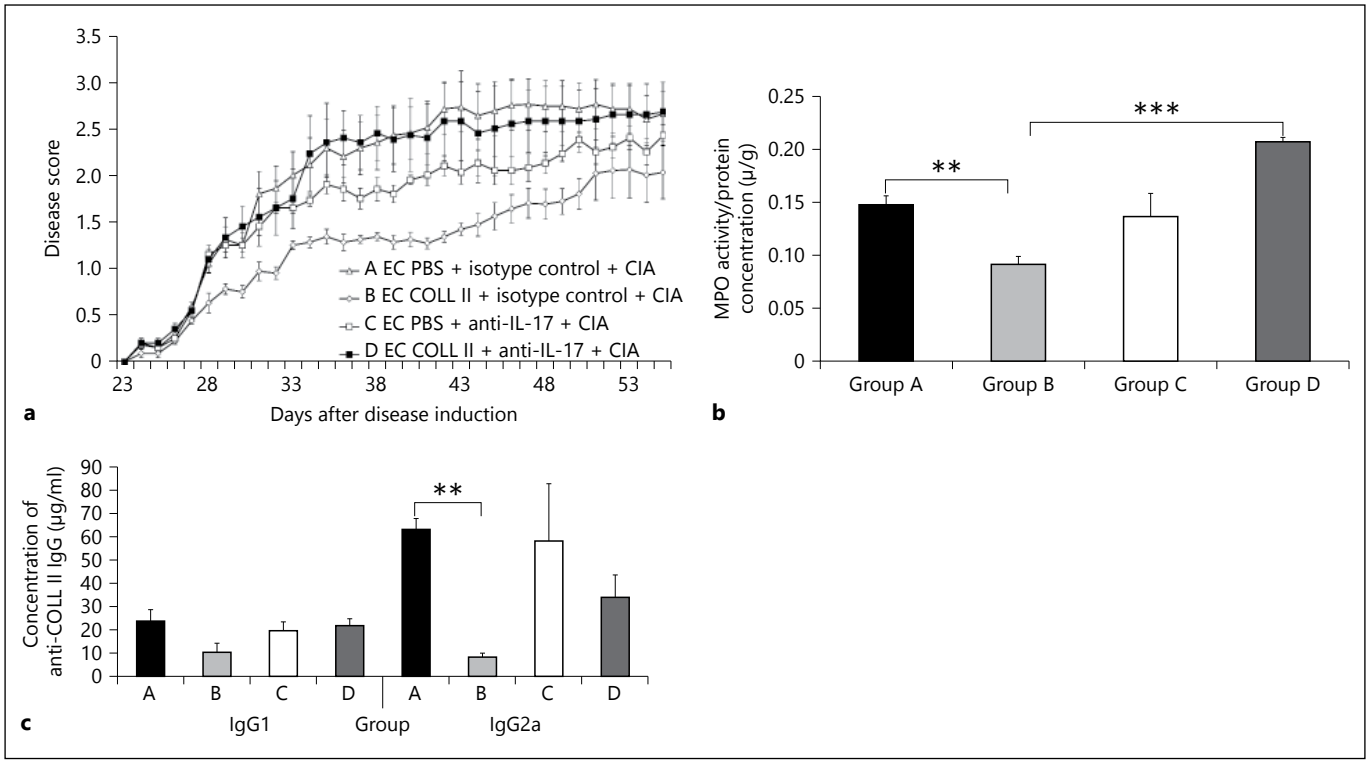

Fig. 8. In vivo neutralization of IL-17 abrogates EC-induced suppression. **a** Mice were EC immunized with COLL II twice and then injected i.p. with 100 μg of anti-IL-17 mAb (group D) or isotype control antibody (group B) 1 day before (day 6) and on the day of patch removal (day 7). Additionally, mice EC treated with PBS alone received two injections of either anti-IL-17 mAb (group C) or isotype control antibody (group A) 1 day before (day 6) and on the day of patch removal (day 7). Next, mice were immunized with COLL II to induce CIA. Each group contained 8–10 mice. Data are presented as the mean arthritis score in each group ± SE. Statistical significance between groups emerged after day 28: $p < 0.05$ for group B versus group A. **b** The level of MPO activity was evaluated in the joint tissue isolated on day 55 after disease induction. Each group contained 4–5 samples. Values are presented as the mean ± SE. ** $p < 0.01$; *** $p < 0.001$. **c** The production of anti-COLL II IgG1 and IgG2a antibodies was measured using ELISA in the mouse serum harvested on day 55 after disease induction. Each group contained 4–6 samples. Values are presented as the mean ± SE. ** $p < 0.01$.

the presence of COLL II. Culture supernatants were tested for the concentration of pro- and anti-inflammatory cytokines. EC immunization with COLL II prior to the induction of CIA (group B) significantly inhibited production of IFN-γ when compared to the group EC treated with PBS (group A), whereas there was no difference in IL-4, IL-6, IL-10, IL-17E and TNF-α production between the tested groups (fig. 7b). The level of IL-17A was significantly increased in mice EC treated with COLL II prior to CIA induction when compared with PBS patched animals before immunization with COLL II plus CFA.

In vivo Neutralization of IL-17 Abrogates EC-Induced Suppression

Our in vitro experiments showed that EC immunization with COLL II results in elevated production of IL-17A. To determine if IL-17A is involved in skin-induced tolerance, mice were EC immunized with COLL II on days 0 and 4, and patches were removed on day 7. The mice were also injected i.p. with 100 μg of anti-IL-17 mAb (group D) or isotype control antibody (group B) 1 day before (day 6) and on the day of patch removal (day 7). Additionally, mice EC treated with PBS alone received two injections of either anti-IL-17 mAb (group C) or isotype control antibody (group A) 1 day before and on the day of patch removal. Then, all four groups of mice were immunized with COLL II to induce CIA according to the protocol described in Materials and Methods. Mice EC immunized with COLL II and treated with the isotype control antibody exhibited CIA of reduced severity when compared to the positive control (group B vs. group A; fig. 8a). In contrast, mice patched with COLL II and treat-

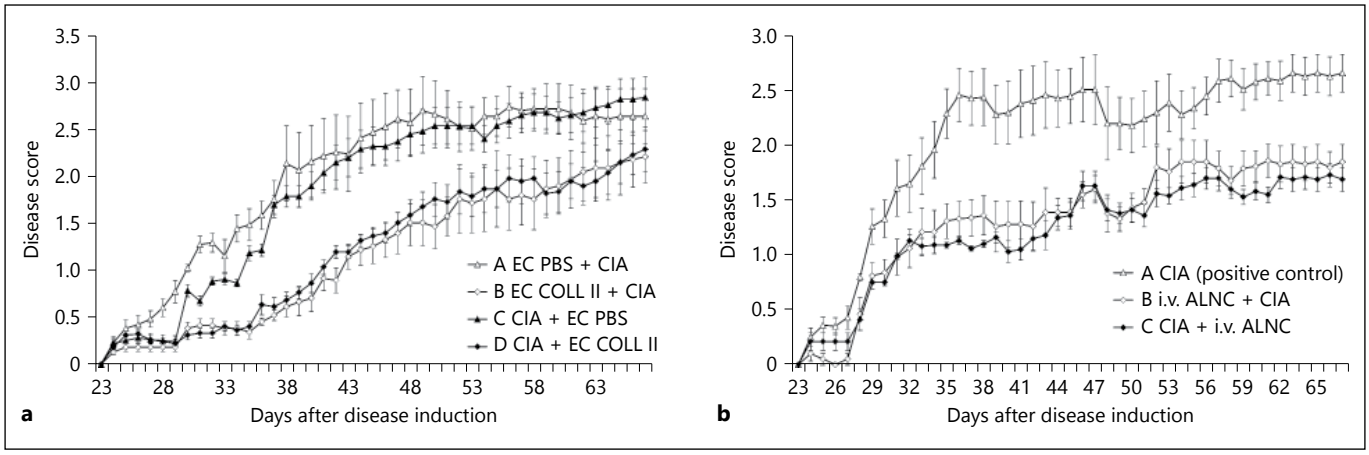

Fig. 9. EC immunization with COLL II alleviates ongoing CIA. **a** EC immunization with COLL II was performed prior to the induction of CIA (group B) or following the onset of CIA (group D). Control groups were treated with PBS before CIA induction (group A) or upon the first signs of disease (group C). Each group contained 6–9 mice. Data are presented as the mean arthritis score in each group ± SE. Statistical significance between groups emerged after day 28: $p < 0.05$ for group B versus group A; $p < 0.01$ for group D versus group A. **b** 2.5×10^7 EC-induced Ts cells were i.v. transferred into mice on the day of CIA induction (group B) or on day 21 after disease induction (group C). The control group did not receive any cells (group A). Each group contained 10 mice. Data are presented as the mean arthritis score in each group ± SE. Statistical significance between groups emerged after day 28: $p < 0.05$ for group B versus group A; $p < 0.01$ for group C versus group A.

ed with anti-IL-17 mAb developed disease that was as severe as in the animals in the positive control (group D vs. group A). Injection of anti-IL-17 mAb into mice EC treated with PBS before CIA induction did not significantly affect the disease course when compared to positive controls (group C vs. group A).

Additionally, macroscopic disease evaluation was fully confirmed by MPO estimation in joint tissues isolated on day 63 after disease induction (fig. 8b) and the production of anti-COLL II IgG1 and IgG2a antibodies (fig. 8c). These data suggest that IL-17A induced via EC immunization with COLL II is involved in the reduction of disease severity.

EC Immunization with COLL II Can Ameliorate Ongoing CIA

Our previous experiments showed that EC immunization with COLL II prior to CIA induction protects from the disease. We then sought to determine whether EC immunization with COLL II could suppress ongoing CIA. This was accomplished by inducing CIA by active immunization with COLL II and CFA. Upon the first signs of disease, the mice were EC exposed to COLL II for 4 days, and then the patches were reapplied for an additional 3 days. EC immunization with COLL II following the onset of CIA suppressed the disease following its peak to the same extent as when mice were tolerized prior to the induction of CIA (group D vs. group B; fig. 9a). EC treatment with PBS either before CIA induction or upon the first signs of disease did not affect disease severity (groups A and C vs. group B).

Another approach was used to confirm the finding that EC immunization with COLL II could suppress ongoing CIA. On the day of CIA induction or on day 21 after disease induction, 2.5×10^7 EC-induced Ts cells were transferred into mice. The results showed that the transfer of Ts cells either before or after CIA induction protects from disease (groups B and C vs. group A; fig. 9b). As depicted in figure 9a and b, disease started before the mice were injected with LPS.

Discussion

In the current study we have demonstrated that EC immunization with COLL II spread over a gauze patch can alleviate the inflammatory response during CIA. EC immunization with COLL II results in a significant reduction of CIA, and the observed protection from disease is dose dependent. Decreased disease severity correlates with reduced MPO activity in joint tissue.

It is well known that posttranslational modifications of proteins play important roles in their biological functions. However, some of these modifications can convert

host proteins into autoreactive forms, which might lead to the induction of autoimmune responses. Some modifications of peptidyl-arginines have been shown to result in a highly specific autoantibody response. Antibodies against citrullinated proteins are unique to patients with autoimmunity such as RA [28]. It has been proposed that both the anti-CCP autoantibodies and their targets are produced locally [29]. Anti-CCP antibodies may occur early in disease, or even before clinical manifestations, which is why these antibodies have a high prognostic value in RA [30]. Studies performed by Kuhn et al. [27] have shown that an autoantibody response to citrullinated proteins, which is indistinguishable from that observed in RA, develops in CIA too. Furthermore, it has been demonstrated that the presence of anti-citrullinated protein antibodies in CIA plays a key role in the development of inflammatory arthritis. Our data confirmed that DBA/1 mice produce a detectable level of anti-CCP antibodies after CIA induction. Additionally, EC immunization with COLL II prior to the induction of CIA decreases the production of anti-CCP antibodies, which correlates with a reduced severity of the disease as well as with decreased production of pathogenic anti-COLL II IgG2a antibodies.

Skin-induced suppression can be adoptively transferred with ALNC and SPLC isolated from mice EC immunized with COLL II. However, neither MLNC nor Thy can transfer EC-induced suppression. These data suggest that EC immunization with COLL II induces suppressor cells that inhibit the inflammatory response in the joints, and that these suppressor cells are present only in peripheral lymph organs, namely inguinal and auxiliary lymph nodes and spleen.

By transferring decreasing numbers of ALNC we find that very low numbers of skin-induced suppressor cells, such as 4×10^5 of whole ALNC per mouse, are able to ameliorate the inflammatory reaction in CIA. These data suggest that EC-induced suppressor cells are very efficient regulators. This is consistent with our previous observation that small numbers of purified TCRαβ$^+$ suppressor cells can ameliorate the inflammatory response, since only 2.5×10^3 sorted cells efficiently inhibits CHS and EAE [15–17].

Negative selection experiments show that the skin-induced suppressor cells belong to the population of TCRαβ$^+$ lymphocytes. It is worth highlighting that depletion of the TCRαβ$^+$ population from ALNC not only abolishes protection from the disease, but even causes its aggravation. It may suggest that TCRγδ$^+$ lymphocytes in the absence of TCRαβ$^+$ suppressor cells can promote an inflammatory response. This is not surprising, as we have already shown that TCRγδ$^+$ can support a Th1-mediated CHS reaction [31] and inflammatory reaction in EAE [32].

Pro- and anti-inflammatory cytokines play roles in EC-induced suppression of CIA. EC immunization with COLL II does not affect the production of IL-4, IL-6, IL-8, IL-10, IL-12, IL-17E, IL-35, TGF-β and IFN-γ when compared to PBS-treated animals. Additionally, experiments employing a transwell system showed that EC-induced suppression does not require direct cell-to-cell contact (data not shown).

Surprisingly, we found that EC immunization with COLL II results in increased production of IL-17A and an increased percentage of TCRαβ$^+$ RORγt$^+$ cells in auxiliary and inguinal lymph nodes. This finding is in line with the data of Geha and colleagues [33] who have shown that EC immunization with OVA induces a Th17 response. In our system, in vivo neutralization of IL-17 with anti-IL-17 mAb abolishes EC-induced protection from the disease. MPO activity in tissue homogenates and production of anti-COLL II IgG2a antibodies macroscopically confirms these data. It has been reported that IL-17A can act in some cases as an anti-inflammatory agent that ameliorates dextran sulfate sodium-induced colitis [34] and suppresses inflammation in a murine model of asthma [35]. Additionally, Flavell and colleagues [36] have shown that IL-17A provides protection in a mouse transfer model of colitis and that, in this experimental system, T cells function both as the source and as the relevant target of IL-17. An anti-inflammatory role of IL-17A has also been shown in experimental autoimmune uveitis [37], *Helicobacter pylori*-induced gastritis [38], experimental diabetes in NOD mice [39] and atherosclerosis [40]. It is well accepted that IL-17A acts as a proinflammatory cytokine in the pathogenesis of autoimmune diseases, including arthritis [41, 42]. More recently, an IL-17A-producing CD161$^+$ FoxP3$^+$ Treg cell population has been characterized [43]. These cells are highly enriched within the inflammatory environment of childhood arthritis [43]. This observation has been confirmed by other groups showing the existence of IL-17A-producing CD4$^+$ CD25^{hi} CD127^{lo} CD161$^+$ CD45RA$^-$ Treg cells in humans [44]. This population of Treg cells is functionally suppressive, has phenotypic/molecular characteristics to other subpopulations of Treg cells, and retains suppressive function following IL-17 induction. These CD161$^+$ FoxP3$^+$ Treg cells accumulate in inflamed joints of patients with inflammatory arthritis and are the predominant IL-17-producing Treg cell population at these sites [44]. These data strongly support our finding that IL-17A

may be involved in the negative regulation of inflammatory responses during CIA. At present it is unclear how IL-17A can suppress CIA after skin patching. However, the finding of Schnyder et al. [45] that IL-17A suppresses the TNF-α-induced vascular adhesion molecule (VCAM)-1 expression implies that IL-17A is not only a strictly pro-inflammatory cytokine, but also exhibits a regulatory function. Thus, in our experimental model, IL-17A may partly inhibit the inflammatory reaction by suppression of T effector cell influx into inflamed tissue. Additionally, we find that EC immunization with COLL II significantly inhibits IFN-γ production in mice with CIA (fig. 7b). Inhibition of IFN-γ production may be a part of the mechanism of EC-induced protection from CIA. Our finding is in line with observations in other laboratories that IL-17A may inhibit the development of Th1 cells and IFN-γ production [38, 40, 46, 47]. Thus, our study shows that IL-17A, apart from its well-established proarthritogenic action [48, 49], may also be a negative regulator of inflammatory responses. Other studies using the CIA model show that treatment of mice with anti-IL-17 antibody at the onset of disease reduced its incidence and severity [50]. Additionally, experiments in a mouse model of asthma show that neutralization of IL-17A may inhibit or aggravate inflammatory response in the lungs [51, 52]. These variabilities may reflect differences in study design such as the time and dose of anti-IL-17 antibody administration. Thus, it is possible that IL-17A plays different roles at various stages of CIA. It has been shown previously that other cytokines can diversely regulate immune response. For instance, high levels of IL-4 can inhibit CHS, whereas a low level of this cytokine is indispensable during the initiation of CHS [53, 54].

Our results, showing that EC immunization with COLL II is an effective strategy to induce a population of suppressor T cells able to protect from CIA, are supported by a study by Strid et al. [55] in which protection is induced in DBA/1-TCR-β Tg transgenic mice bearing a TCR specific for COLL II. However, there are important differences in the mechanism of tolerance in our study and their report. We find that IL-17A has a protective role in EC-induced tolerance, while Strid et al. [55] suggest that the Th2-type cytokine IL-4 might be responsible for the observed suppression. We attribute this difference to the distinct techniques of EC immunization. In our study, EC immunization was performed by applying a gauze patch soaked with COLL II to the shaved skin of the mouse dorsum, whereas Strid et al. [55] applied antigen to the ear skin after the stratum corneum has been removed. As this group has shown previously, this technique of EC immunization promotes a Th2 response [56]. There is also a difference in the time of immunization used in both studies. In our case, mice are EC exposed to COLL II for 1 week before disease induction, whereas Strid et al. [55] EC immunize mice 3 weeks prior to disease induction, EC expose to COLL II on 3 consecutive days, and boost 2 weeks after the initial exposure to antigen. In addition, conditions in DBA/1 wild-type and DBA/1-TCR-β Tg mice may favor the induction of different populations of T cells that suppress inflammatory responses.

Furthermore, our work shows that skin-induced suppression of CIA declines with time and persists up to 4 weeks after patch removal. We have made similar observations in the CHS model [14, 57]. This may result from either a limited lifespan of skin-induced suppressor cells or the disappearance of antigen from the organism. The observed attenuation of skin-induced suppression suggests that for a prolonged therapeutic effect a booster treatment might be required.

In summary, induction of tolerance via EC immunization with protein antigen can inhibit cell-mediated immune responses in an animal model of RA, as we have previously demonstrated in CHS [14, 15], allogeneic skin graft rejection [19] and TNBS-induced colitis [18], as well as in an animal model of multiple sclerosis [16, 17, 58] and in patients with multiple sclerosis [20, 21]. This method may therefore be useful for the treatment of a variety of inflammatory diseases. Immunization via EC application of a protein antigen that induces Ts cells to inhibit inflammatory responses may become an attractive, noninvasive, needle-free therapeutic method for different clinical situations.

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