Targeting of the Transcription Factor STAT4 by Antisense Phosphorothioate Oligonucleotides Suppresses Collagen-Induced Arthritis

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The transcription factor STAT4 mediates signals of various proinflammatory cytokines, such as IL-12, IL-15, and IL-23, that initiate and stabilize Th1 cytokine production. Although Th1 cytokine production has been suggested to play a major pathogenic role in rheumatoid arthritis, the role of STAT4 in this disease is poorly understood. In this study, we demonstrate a key functional role of STAT4 in murine collagen-induced arthritis (CIA). In initial studies we found that STAT4 expression is strongly induced in CD4⁺ T cells and to a lesser extent in CD11b⁺ APCs during CIA. To analyze the role of STAT4 for arthritis manifestation, we next investigated the outcome of interfering with STAT4 gene expression in CIA by using STAT4-deficient mice. Interestingly, STAT4-deficient mice developed significantly less severe arthritis than wild-type control mice and the T cells from such mice produced less IL-6, TNF, and IL-17. In addition, the targeting of STAT4 expression by a specific antisense phosphorothioate oligonucleotide directed at the translation start site suppressed STAT4 levels and signs of CIA even when applied during the onset of disease manifestation. These data suggest a key regulatory role of STAT4 in the pathogenesis and manifestation of murine collagen-induced arthritis. Furthermore, the targeting of STAT4 emerges as a novel approach to therapy for chronic arthritis. The Journal of Immunology, 2007, 178: 3427–3436.
processes in the pathogenesis of CIA is less clear because there are conflicting data with regard to this issue. The analysis of the contribution to CIA of either of the two IL-12-forming subunits, p40 and p35, using IL-12 p40−/− and IL-12 p35−/− deficient mice, respectively, revealed that p35−/− mice are highly susceptible whereas p40−/− mice show a strikingly different phenotype with the suppression of disease activity (17). Similar results were obtained when p40−/− and p35−/− mice were compared after induction of the T cell-mediated murine experimental autoimmune encephalitis model (EAE) (18). These data implied that another factor different from the IL-12 p70 heterodimer shares the p40 subunit and contributes to proinflammatory disease activity mediated by Th1-like T cells. Subsequently, Oppmann et al. (19) described a new cytokine, IL-23, which consists of a heterodimer of p40 and the newly identified subunit p19. IL-23 is mainly produced by dendritic cells and macrophages and preferably acts on memory T cells promoting their proliferation and the production of IL-17 (20, 21). In analogy to IL-12 signaling, Parham et al. (22) could demonstrate that IL-23 binding to the IL-23R complex leads to activation of the Jak/STAT cascade as well as to activation of STAT4.

Considering the conflicting data in terms of the role of IL-12 in the pathogenesis of CIA, recent reports suggest the possibility that the existence of intact IL-23 rather than IL-12 is crucial for the development of T cell-mediated proinflammatory disease stages (17, 23) and that both IL-12 and IL-23 mediate the activation of STAT4. We therefore addressed the role of STAT4 in CIA by exploiting two approaches. First, we studied the consequences of STAT4 deficiency on the outcome of CIA in vivo and observed a significantly reduced incidence and severity of disease manifestations in mice lacking STAT4. Second, we investigated the in vivo effects of an antisense (AS) DNA strategy against the translational start site of STAT4 on the clinical course of CIA. These data implied that cytokine-induced STAT4 activation exerts its arthritis-promoting effects especially during the phase of arthritis manifestation. Hence, targeting STAT4 emerges as an interesting target for the development of novel RA therapeutics in the future.

Materials and Methods

Mice

STAT4-deficient BALB/c mice (24) were purchased from The Jackson Laboratory and backcrossed five times in the DBA/2 background for arthritis experiments. Genotyping was performed by PCR using ear biopsy-derived DNA with the following primer sets: STAT4 wild-type (WT) sense, 5′-CCAACCTCAGACGCAACT-3′; STAT4 WT AS, 5′-GCTCTTTTGAGCAGGGATG-3′; Neo sense, 5′-CTTTGGTGAGGAGGCTAT-3′; and Neo AS, 5′-AGGTTGAGTACGAAGAGGATC-3′. Furthermore, mice used in CIA experiments were homozygous for I-Aq as derived DNA with the following primer sets: STAT4 wild-type (WT) sense, 5′-CCAACCTCAGACGCAACT-3′; STAT4 WT AS, 5′-GCTCTTTTGAGCAGGGATG-3′; Neo sense, 5′-CTTTGGTGAGGAGGCTAT-3′; and Neo AS, 5′-AGGTTGAGTACGAAGAGGATC-3′. STAT4−/− mice were derived from the animal facility of the University of Mainz. In all experiments only male mice are highly susceptible whereas p40−/− mice show a strikingly different phenotype with the suppression of disease activity (17). Similar results were obtained when p40−/− and p35−/− mice were compared after induction of the T cell-mediated murine experimental autoimmune encephalitis model (EAE) (18). These data implied that another factor different from the IL-12 p70 heterodimer shares the p40 subunit and contributes to proinflammatory disease activity mediated by Th1-like T cells. Subsequently, Oppmann et al. (19) described a new cytokine, IL-23, which consists of a heterodimer of p40 and the newly identified subunit p19. IL-23 is mainly produced by dendritic cells and macrophages and preferably acts on memory T cells promoting their proliferation and the production of IL-17 (20, 21). In analogy to IL-12 signaling, Parham et al. (22) could demonstrate that IL-23 binding to the IL-23R complex leads to activation of the Jak/STAT cascade as well as to activation of STAT4.

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Induction of CIA

For arthritis induction, mice were immunized intradermally (i.d.) in the ear and at the base of the tail with 100 μg of CII (Sigma-Aldrich) emulsified in CFA (Difco) at day 0. An i.p. booster injection with 50 μg of collagen alone in PBS followed at day 21.

The mice were visually checked for the appearance of arthritis and were considered to be arthritic when either redness or swelling of one or more digits or other parts of the paw were macroscopically detectable. The severity of arthritis was graded in a semiquantitative manner on a scale from 0 (no signs) to 4 (maximal signs of inflammation) for each paw separately:

0, no symptoms; 0.5, redness or swelling of one digit; 1, swelling and erythema of two digits; 2, swelling of more than two digits or mild swelling of the limb; 3, significant but not maximal swelling of the complete or almost complete paw; 4, maximal swelling and erythema of the entire limb and later merging into ankylosis. The scores of four paws per mouse were added so that the maximal possible score per mouse was 16.

Histological examination

Mice were killed at indicated time points followed by the removal of hind paws and fixation of the samples in 10% formic acid for at least 12 h. After decalcification in EDTA, the specimens were embedded in paraffin and stained with H&E. The histopathological grading of joint lesions was performed semiquantitatively on a scale from 0 to 3. The following criteria were examined: inflammatory infiltrate (0, none; 1, mild; 2, moderate; 3, severe infiltrate); synovial lesion (0, no lesion; 1, mild alteration; 2, moderate alteration; 3, severe/complete destruction of the synovia); cartilage destruction (0, none; 1, mild; 2, moderate; 3, severe destruction with loss or complete fragmentation of cartilage); and bone destruction (0, none; 1, mild destruction of subchondral bone; 2, moderate destruction; 3, severe destruction with loss of large areas of bone).

The histological grading was done in a blinded fashion by the same pathologist (P.S.) (25). The total scores represent the mean values of all single scores (mean ± SD) of histologically diseased individual mice within the indicated group.

Cytokine production of splenocytes derived from immunized mice

IFN-γ, TNF-α, and IL-6 production by bulk splenocytes from STAT4−/− and STAT4+/+ mice was measured ex vivo at the indicated time points (i.d. on days 10, 40, and 56) after immunization. Briefly, spleens were removed and a single-cell suspension was generated by pushing the spleen through a sterile 40-μm cell strainer. After removal of erythrocytes from the cell suspension by hypotonic lysis in ACK (ammonium chloride potassium phosphate) buffer, spleen cells were washed in PBS and consecutively cultured in 24-well plates with the indicated amounts of CII, 10 μg/ml murine IL-12 (PeproTech) or remained unstimulated in RPMI 1640 medium (BioWhittaker) supplemented with 10% heat-inactivated FCS, 3 mM L-glutamine, 10 μg/ml HEPES buffer, and 100 U/ml penicillin and streptomycin (BioWhittaker). Where indicated, 24-well plates were coated with 10 μg/ml hamster anti-mouse CD3 Ab (clone 145-2C11) (BD Pharmingen) overnight at 4°C, and unbound Abs were removed by washing with PBS before use. After 24 h, supernatants were collected and either cytokine ELISA analysis was performed immediately as described below or the supernatants were stored at −20°C for later analysis.

ELISA

Cytokine concentrations in supernatants were measured by sandwich ELISA. Briefly, 96-well plates (Maxisorb; Nunc) were coated overnight at 4°C with 3 μg/ml (IFN-γ), 2 μg/ml (IL-6), or 800 ng/ml (TNF-α) of monoclonal anti-mouse Abs in PBS (100 μl/well). After washing the plates three times with washing buffer (PBS with 0.05% Tween 20; Sigma-Aldrich), wells were blocked with 200 μl/well blocking buffer (PBS, 1% BSA, 5% sucrose, and 0.05% NaCN) for 1 h at 37°C followed by three washes with washing buffer. Serial dilutions of samples and standards in PBS and 1% BSA (in the case of TNF-α, BSA with 0.1% BSA) were transferred to ELISA plates (100 μl/well) and incubated overnight at 4°C. Plates were washed three times and diluted polyclonal biotinylated Abs for each cytokine were added for IFN-γ, 200 ng/ml for IL-6; 300 ng/ml for TNF-α in 100 μl/well TBS and 0.1% BSA for 2 h at room temperature. After three washes with PBS and 0.05% Tween 20, 100 μl of streptavidin-peroxidase (DakoCytomation) in a dilution of 1/1000 in PBS and 1% BSA was added to each well and incubated for 30 min at room temperature. Plates were then washed an additional three times followed by the addition of 100 μl/well substrate buffer containing 3,3′,5,5′-tetramethylbenzidine. The color reaction was abrogated by adding 50 μl/well sulfuric acid. The colored product was measured at a wave length of 450 nm using an EMAX precision microplate reader (Molecular Devices). All Abs and standard proteins were purchased from R&D Systems. The IL-23 ELISA was purchased from NatuTec and performed according to the manufacturer’s directions.

Cytokine detection by flow cytometry

As indicated in some experiments, cytokine levels in cell culture supernatants were measured using the FlowCytomix mouse Th1/Th2 cytokine multiplex kit (Bender MedSystems; catalog no. BMS820FF) according to the manufacturer’s directions.
In the CIA experiments, 8- to 12-wk-old male DBA/1 mice were immunized with CII in CFA (i.d. on day 0 and boosted on day 21 with CII in PBS i.p.) according to the protocol described above. On day 26 after immunization, mice received an i.v. treatment with 800 µg of STAT4 AS oligonucleotides. Control animals were either treated with 800 µg of STAT4 MM oligonucleotides or with the solvent PBS alone (total volume, 200 µl). Treatment with 400 µg DNA (STAT4 AS or MM) or PBS injection i.v. was repeated on day 33. Arthritis development was checked by inspection at the indicated time points and scored as described above. At day 40 after immunization, the mice were killed and hind paws were removed for histological examination as described above.

**Purification of CD4⁺ and Thy1.2⁺ splenocytes by magnetic beads**

In some experiments, splenocytes derived from immunized mice were sorted using magnetic beads (MACS) to enrich for CD4⁺ and to separate Thy1.2⁺ (CD90) from Thy1.2⁻ cells according to the manufacturer’s directions (Miltenyi Biotec).

**Western blotting**

Whole cell lysates from CD4⁺ and CD11b⁺-enriched splenocytes derived from immunized DBA/1 WT mice were generated and Western blot analysis was performed using a standard protocol. Briefly, 30 µg protein was used per lane and separated by electrophoresis on a 7.5% SDS polyacrylamide gel. After wet blotting onto nitrocellulose, the membrane was blocked in PBS with 0.2% Tween 20 and 5% nonfat dry milk at 37°C for 1 h. Then, the blots were incubated with rabbit anti-STAT4 (Santa Cruz Biotechnology) at 4°C overnight, washed at least three times in PBS with 0.2% Tween 20, and subsequently incubated with HRP-conjugated anti-rabbit IgG (code P0217; DakoCytomation) in PBS with 0.2% Tween 20 and 5% nonfat dry milk for 5 h at room temperature. After three washes with PBS and 0.3% Tween 20 the final reaction was developed using a chemiluminescent system (ECL; Amersham Biosciences). For reprobing, the blots were stripped in 0.2M glycine (pH 2.8). All blots were reprobed with anti-β-actin and HRP (Santa Cruz Biotechnology) for normalization and developed as described above.

**EMSA**

CD4⁺ T cells were isolated from naive splenocytes using Dynal beads and transfected with STAT4 AS, STAT4 MM, or unrelated oligonucleotides preincubated with Lipofectamine. After stimulation with 10 U/ml rIL-12 (Genzyme) for 48 h the cells were harvested, nuclear proteins were extracted, and a gel retardation assay was performed using a STAT4-specific DNA binding site that has been previously shown to bind to STAT4 (26, 27). The binding reaction was performed at room temperature for 30 min. After electrophoresis, the gels were dried and exposed to Kodak MS films on intensifying screens at ~80°C. The sequences of the oligonucleotides for EMSA, which were sequenced, are shown in Supplemental Table I. The blots were reprobed with anti-STAT4 (Santa Cruz Biotechnology) at 4°C overnight, washed at least three times in PBS with 0.2% Tween 20, and subsequently incubated with HRP-conjugated anti-rabbit IgG (code P0217; DakoCytomation) in PBS with 0.2% Tween 20 and 5% nonfat dry milk for 5 h at room temperature. After three washes with PBS and 0.3% Tween 20 the final reaction was developed using a chemiluminescent system (ECL; Amersham Biosciences). For reprobing, the blots were stripped in 0.2M glycine (pH 2.8). All blots were reprobed with anti-β-actin and HRP (Santa Cruz Biotechnology) for normalization and developed as described above.

**Cytospin**

Naïve CD11b⁺ splenocytes from BALB/c WT mice were isolated by MACS and transfected for 3.5 h with fluorescein-labeled STAT4 AS oligonucleotides (16 µM) preincubated with Lipofectamine as described above. After culturing the cells for 18 h in the presence of 1 µg/ml LPS, cytospins of these cells were generated and analyzed by fluorescence microscopy. In brief, after 18 h of incubation the cells were washed twice in RPMI 1640 and then resuspended in medium at 10⁶ cells/ml. Cells (10⁵) were transferred on cytocentrifuge slides and spun for 5 min at 500 rpm. After air drying of the slides, the nuclei were counterstained with Hoechst dye (blue) diluted 1/1000 in PBS for 30 min at room temperature. The slides were washed several times in PBS and 0.1% Tween 20 and mounted in one drop of mounting medium (Vector Laboratories).

**Results**

**STAT4 deficiency leads to a marked reduction of the incidence and severity of CIA**

To gain insight into the contribution of STAT4 for the development of arthritis, we generated arthritis-susceptible STAT4⁻/⁻ BALB/c mice five times
experiments were performed showing similar results. Within the particular group. The results represent pooled data of two independent experiments with at least four mice per group. In total, four independent data shown here display the pooled results of four independent experiments.

of all single scores (mean scale of the morphological changes of an individual mouse ranged from 0 (no changes) to 3 (severe changes). The total scores represent the mean values of an individual mouse (maximal score per mouse was 16) at the indicated time points. The arthritis index indicates mean scores ± SD of the arthritic mice within the particular group. The results represent pooled data of two independent experiments with at least four mice per group. In total, four independent experiments were performed showing similar results. B, STAT4−/− mice display strikingly reduced histological signs of arthritis whereas histologically there were no detectable differences between STAT4+/+ and STAT4−/− mice. STAT4+/+, STAT4−/−, and STAT4+/− mice were immunized as described in A. Fifty-six days after immunization, the hind paws of indicated groups were removed and paraffin sections were stained with H&E. The histological analysis of sections from immunized STAT4−/−, STAT4+/−, and STAT4+/+ mice was performed in a blinded fashion (see Materials and Methods). The scale of the morphological changes of an individual mouse ranged from 0 (no changes) to 3 (severe changes). The total scores represent the mean values of all single scores (mean ± SD) of histologically diseased individual mice within the particular group. The results include at least 14 mice per group. The data shown here display the pooled results of four independent experiments. C, Production of proinflammatory cytokines by STAT4−/− splenocytes is strikingly reduced compared with STAT4 WT splenocytes. IFN-γ, TNF-α, and IL-6 production by bulk splenocytes from STAT4−/− and STAT4+/+ mice were measured ex vivo on day 56 after immunization. Splenocytes were restimulated in vitro with either 50 μg/ml CII or 10 U/ml IL-12 or remained unstimulated for 24 h. IFN-γ, TNF-α, and IL-6 production were measured in the supernatants by ELISA. The data represent results from one of two independently performed experiments with similar results and show mean values ± SD of at least three mice per group. D, Reduced production of arthritis-promoting cytokines by STAT4-deficient T cells whereas IL-23 production of non-T cells is indistinguishable between WT and STAT4−/− mice. Mouse splenocytes of the indicated genotype were magnetically separated in Thy1.2+ and Thy1.2− fractions and activated for 48 h in vitro. IL-6, TNF-α, IFN-γ, IL-17, and IL-10 were measured in cell culture supernatants by flow cytometric bead array and IL-23 was measured by ELISA. The cytokine levels of STAT4−/− cells are shown as percentages in relation to the cytokine production of WT mice (defined as 100%). The results display mean values ± SEM of at least eight individual mice per genotype from two independent experiments.

into the DBA/1 background. The induction of CIA and the monitoring for clinical signs of arthritis was performed as specified in Materials and Methods. In all arthritis experiments only male, 8- to 12-wk-old STAT4−/−, STAT4+/−, and STAT4+/+ mice homozygous for MHC class II molecule H-2k were used for immunization.

Interestingly, STAT4−/− mice were almost completely resistant to arthritis induction. Fig. 1A displays the pooled results of two independent, representative experiments in which STAT4-deficient mice had little or no signs of disease. As shown in Table I, only one of 23 collagen-immunized STAT4−/− mice (4%) developed macroscopic signs of arthritis (onset day 27, maximum score 8). STAT4+/− heterozygous mice had a delayed onset (mean day 45 ± 11.8 vs day 36 ± 9.6 in WT mice) and a reduced incidence of arthritis (44 vs 62% in WT mice) (Table I). Once afflicted, however, these mice showed clinical signs of arthritis comparable to those of WT mice (arthritis index 5.31 in STAT4+/− vs 4.94 in WT mice). In addition, there was no difference between fifth generation STAT4+/+ mice originating from backcrossing and DBA/1 inbred mice in terms of incidence and severity of arthritis development.
**Table I. Diminished clinical signs of CIA in STAT4−/− mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Incidence of Disease</th>
<th>Day of Onset</th>
<th>Arthritis Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT4−/+</td>
<td>18/29 (62%)</td>
<td>36 ± 9.6</td>
<td>4.94 ± 3.52</td>
</tr>
<tr>
<td>STAT4+/−</td>
<td>8/18 (44%)</td>
<td>45 ± 11.8</td>
<td>5.31 ± 3.32</td>
</tr>
<tr>
<td>STAT4−/−</td>
<td>1/23 (4%)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* CIA was induced in STAT4+/−, STAT4−/−, and STAT4+/+ mice as described in Materials and Methods. Disease development was determined by inspection at the indicated time points and scored as described in Materials and Methods. The table shows the overall incidence of disease compared with the total number of initially immunized mice, the day of onset (mean ± SD), and the arthritis index representing the mean scores ± SD of arthritic mice. The results represent the pooled data of five independent experiments with the indicated total numbers of mice.

**Table II. Comparisons of histological differences between STAT4+/−, STAT4−/−, and STAT4+/+ mice after arthritis induction**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Incidence of Disease</th>
<th>Clinic</th>
<th>Histology</th>
<th>Rangea</th>
<th>Inflammatory Infiltratea</th>
<th>Synovial Lesionsaa</th>
<th>Cartilage Destructionaa</th>
<th>Bone Destructionaa</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT4+/−</td>
<td>11/21</td>
<td>11/21</td>
<td>1–3</td>
<td>2.09 ± 0.70</td>
<td>2.18 ± 0.75</td>
<td>2.27 ± 0.65</td>
<td>2.27 ± 0.65</td>
<td></td>
</tr>
<tr>
<td>STAT4+/−</td>
<td>7/14</td>
<td>7/14</td>
<td>1–3</td>
<td>2.14 ± 0.9</td>
<td>2.29 ± 0.76</td>
<td>1.86 ± 0.69</td>
<td>2.2 ± 0.75</td>
<td></td>
</tr>
<tr>
<td>STAT4−/−</td>
<td>1/18</td>
<td>4/18</td>
<td>0.5–1</td>
<td>0.5 ± 0</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0 ± 0</td>
<td></td>
</tr>
</tbody>
</table>

* Fifty-six days after arthritis induction the hind paws of the indicated genotypes were removed and paraffin sections were stained with H&E. The sections were analyzed and compared the cytokine production at day 56 after immunization. Splenocytes were restimulated ex vivo with either 50 μg/ml CIA or 10 U/ml IL-12 or remained unstimulated for 24 h. Production of IFN-γ, TNF-α, and IL-6 was measured in the supernatants by ELISA. As shown in Fig. 1C, STAT4−/− splenocytes did not produce significant amounts of IFN-γ in response to either stimuli or under unstimulated conditions, consistent with a role for STAT4 in controlling IFN-γ production of T cells. In contrast, STAT4 WT T cells produced marked amounts of IFN-γ upon Ag-specific stimulation. In addition, STAT4 deficiency led to decreased levels of both TNF-α and IL-6 in comparison with the levels produced by STAT4 WT splenocytes after restimulation with collagen ex vivo at day 56.

To further discriminate between T cell- and non-T cell-derived cytokine expression profiles, we separated Thy1.2+ T cells and Thy1.2− splenocytes from collagen-immunized STAT4 WT and STAT4-deficient mice. Interestingly, anti-CD3/anti-CD28 Ab plus IL-12 stimulated Thy1.2+ T lymphocytes from collagen-immunized STAT4−/− mice did not only produce reduced levels of IFN-γ, IL-6, and TNF-α but also significantly lower amounts of IL-17 as compared with WT control lymphocytes (Fig. 1D). In contrast, IL-23 production of Thy1.2− cells was indistinguishable between STAT4−/− or STAT4+/+ mice (Fig. 1D). STAT4 thus regulates the production of a panel of T cell-derived proinflammatory cytokines with known pathogenic roles in CIA.

**STAT4 regulates IFN-γ but not IL-6 and TNF production during early phases of CIA**

The above data suggested that the reduced clinical signs of arthritis in STAT4−/− mice might be mediated at least partially by a diminished supply of proinflammatory cytokines that are known to contribute to arthritis manifestation. However, it remained to be determined whether the disease-modifying effect of STAT4 deficiency is, in addition, due to an impaired Ag priming. To directly address this early step in the course of CIA, STAT4−/− and STAT4+/+ male mice were immunized i.d. with collagen in CFA on day 0. On day 10, spleens were removed and splenocytes were restimulated ex vivo with increasing dosages of collagen as indicated or remained unstimulated for 24 h. Supernatants were analyzed for the production of indicated cytokines by ELISA (Fig. 2). Similar to the above results, there was a marked impairment of IFN-γ production by STAT4−/− splenocytes as compared with WT cells. However, there was no difference in the capacity of TNF-α or IL-6 production between WT and STAT4-deficient mice on day 10, suggesting that the differences observed above at later stages of disease are secondary to the STAT4 effects on IFN-γ production during early phases of CIA. In addition, we performed proliferation assays with splenocytes derived from the same experimental setting. After 54 h, cells were pulsed with [3H]thymidine for an additional 18 h. As shown in Fig. 2, both STAT4−/−...
CD11b indicated time points the spleens were removed and the splenocytes of individual mice were further processed by magnetic cell separation, isolating manifest. Male DBA/1 WT mice were immunized i.d. with CII emulsified in CFA at day 0 and boosted with collagen in PBS i.p. at day 21. At the arthritis manifestation. Levels of STAT4 protein are significantly increased in both CD4+ and CD11b+ splenocytes derived from mice at the onset of clinical disease manifestation. Male DBA/1 WT mice were immunized i.d. with CII emulsified in CFA at day 0 and boosted with collagen in PBS i.p. at day 21. At the indicated time points the spleens were removed and the splenocytes of individual mice were further processed by magnetic cell separation, isolating CD11b+ splenocytes followed by isolation of CD4+ cells from the remaining cell suspension. Whole cell protein extracts of these two cell populations were generated and then Western blotting with a STAT4-specific Ab was performed. As shown in Fig. 3, STAT4 protein expression was significantly increased starting on day 27 after arthritis induction preferentially in CD4+ T cells and to a lesser extent in CD11b+ splenocytes. Such an increase was followed by a reduction of STAT4 levels in T cells starting at days 30 and 32 of CIA. Moreover, we observed a second period of increased STAT4 protein expression in CD4+ splenocytes starting at day 37. Taken together, the data show that in the pathogenesis of arthritis, splenic T cells express STAT4 in response to Ag recall in a temporally ordered, biphasic manner.

STAT4 expression is specifically suppressed by a STAT4 AS phosphorothioate oligonucleotide targeting the translation start site of STAT4

Because the above data suggested that the STAT4 protein is specifically expressed during the manifestation period of arthritis and might exert a disease-modulating function in CIA pathogenesis, we next aimed at specifically suppressing STAT4 expression in vivo. Accordingly, we designed several phosphorothioate-modified AS oligonucleotide sequences directed to the translational start site of STAT4 and screened these oligonucleotides in vitro for their ability to diminish STAT4 mRNA levels and STAT4 protein expression.

**FIGURE 2.** Limited effects of STAT4 deficiency on cytokine production and proliferation of splenocytes during the primary response to collagen ex vivo. STAT4+/− splenocytes show abrogated IFN-γ but normal levels of TNF-α and IL-6 production and unaffected proliferative capacity in response to early ex vivo restimulation with CII. STAT4−/− and STAT4+/− male mice were immunized i.d. with CII in CFA on day 0. On day 10, the spleens were removed and the splenocytes were ex vivo restimulated with increasing dosages of CII as indicated or remained unstimulated (US). As a control, splenocytes of naive, unimmunized mice were also included (STAT4+/− w/o CIA). After 54 h [3H]thymidine was added to the cell culture for additional 18 h. After 72 h the [3H]Tdr incorporation was measured. The results represent mean values ± SD of four mice per group from one experiment. Two independently performed experiments showed very similar results.

**FIGURE 3.** Biphasic up-regulation of STAT4 protein expression in splenic CD4+ T lymphocytes during onset and progression of CIA

STAT4 is known to be critical for the differentiation of T cells in Th1 effector cells and is also expressed by both human dendritic cells and monocytes in response to stimulation (28). To further address the question of which types of cells express STAT4 during CIA, we analyzed STAT4 protein expression during the course of CIA in WT DBA/1 mice. At the indicated time points after the immunization of male DBA/1 WT mice, spleens were removed and CD11b+ and CD4+ splenocytes were isolated using immunomagnetic beads (Fig. 3). Whole cell protein extracts of these two cell populations were generated and then Western blotting with a and STAT4+/− splenocytes from collagen-immunized mice proliferated in response to collagen to a similar degree and to a greater extent as compared with the control splenocytes derived from non-immunized DBA/1 WT mice. However, there was no difference in proliferation between STAT4+/− and STAT4+/+ splenocytes. Taken together, these data suggested that there is no relevant STAT4-dependent impairment of the primary response to collagen in immunized mice in terms of the proliferative capacity and production of disease-promoting proinflammatory cytokines, with the important exception of a strikingly decreased IFN-γ production.

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STAT4 is known to be critical for the differentiation of T cells in Th1 effector cells and is also expressed by both human dendritic cells and monocytes in response to stimulation (28). To further address the question of which types of cells express STAT4 during CIA, we analyzed STAT4 protein expression during the course of CIA in WT DBA/1 mice. At the indicated time points after the immunization of male DBA/1 WT mice, spleens were removed and CD11b+ and CD4+ splenocytes were isolated using immunomagnetic beads (Fig. 3). Whole cell protein extracts of these two cell populations were generated and then Western blotting with a and STAT4+/− splenocytes from collagen-immunized mice proliferated in response to collagen to a similar degree and to a greater extent as compared with the control splenocytes derived from non-immunized DBA/1 WT mice. However, there was no difference in proliferation between STAT4+/− and STAT4+/+ splenocytes. Taken together, these data suggested that there is no relevant STAT4-dependent impairment of the primary response to collagen in immunized mice in terms of the proliferative capacity and production of disease-promoting proinflammatory cytokines, with the important exception of a strikingly decreased IFN-γ production.

**Biphasic up-regulation of STAT4 protein expression in splenic CD4+ T lymphocytes during onset and progression of CIA**

STAT4 is known to be critical for the differentiation of T cells in Th1 effector cells and is also expressed by both human dendritic cells and monocytes in response to stimulation (28). To further address the question of which types of cells express STAT4 during CIA, we analyzed STAT4 protein expression during the course of CIA in WT DBA/1 mice. At the indicated time points after the immunization of male DBA/1 WT mice, spleens were removed and CD11b+ and CD4+ splenocytes were isolated using immunomagnetic beads (Fig. 3). Whole cell protein extracts of these two cell populations were generated and then Western blotting with a and STAT4+/− splenocytes from collagen-immunized mice proliferated in response to collagen to a similar degree and to a greater extent as compared with the control splenocytes derived from non-immunized DBA/1 WT mice. However, there was no difference in proliferation between STAT4+/− and STAT4+/+ splenocytes. Taken together, these data suggested that there is no relevant STAT4-dependent impairment of the primary response to collagen in immunized mice in terms of the proliferative capacity and production of disease-promoting proinflammatory cytokines, with the important exception of a strikingly decreased IFN-γ production.
We further investigated the functional capacity of these oligonucleotides to interfere with STAT4 mRNA levels. For this purpose, splenocytes were transiently transfected with STAT4 MM or AS oligonucleotides and stimulated with IL-12 for 6 h followed by RNA isolation and STAT4 mRNA real-time PCR analysis. A phosphorothioate-modified STAT4 AS oligonucleotide led to an ~70% reduction of STAT4 mRNA levels in STAT4 AS oligonucleotide-transfected splenocytes in comparison with untreated controls (Fig. 4B). In contrast, a STAT4 MM oligonucleotide (STAT4 MM) did not result in a marked reduction of STAT4 mRNA level in transfected splenocytes.

Next, we addressed the question whether STAT4 AS treatment would also influence the extent of STAT4 protein expression and especially reduce the STAT4 protein/DNA interaction. In these studies, we isolated CD4+ T cells and treated these cells with STAT4 AS, STAT4 MM, or completely unrelated oligonucleotides in the presence of rIL-12 for 48 h. After extracting nuclear proteins we performed gel-retardation assays (EMSA) using a consensus STAT4 DNA binding site. As shown in Fig. 4C, the coincubation of CD4+ T cells with specific STAT4 AS oligonucleotides led to a significant reduction of STAT4 binding to a STAT4 reference DNA binding site, indicating reduced amounts of STAT4 in the nucleus under STAT4 AS treatment. The addition of a STAT4-specific Ab to STAT4 consensus site resulted in a reduction of the protein/DNA complex, indicating the presence of STAT4 in this complex (data not shown).

To demonstrate efficient transfection and appropriate localization of oligonucleotides, magnetically sorted CD11b+ splenocytes from naïve DBA/1 mice were transiently transfected with fluorescein-labeled STAT4 AS (green) oligonucleotides (24 μM), preincubated with Lipofectamine, and cultured in the presence of 1 μg/ml LPS for 18 h. After 18 h, cytopsins of these cells were generated and analyzed by fluorescence microscopy without (left panel) or with (right panel) nuclear counterstaining using Hoechst dye (blue). The majority of the cells stained were successfully transfected as indicated by a double-positive nuclear staining pattern. B, STAT4 AS oligonucleotide treatment diminishes STAT4 mRNA levels in splenocytes. Unseparated splenocytes from naïve DBA/1 WT mice were transiently transfected with 16 μM STAT4 AS or MM oligonucleotides in complex with Lipofectamine and then coincubated with recombinant murine IL-12 (10 U/ml) for 6 h. As controls, untransfected stimulated (ST) or unstimulated (US) cells were included. After 6 h of coincubation, total RNA was isolated and a real-time PCR analysis of STAT4 expression normalized to the GAPDH expression was performed. The results are shown as percentages in relation to the stimulated untransfected control (defined as 100%) and display mean values ± SD of three independent experiments. C, Nuclear extracts derived from STAT4 AS oligonucleotide-treated CD4+ splenocytes contain less STAT4 protein as indicated by a reduced binding capacity to a STAT4 consensus binding site, whereas treatment with control oligonucleotides has no effect on STAT4 DNA interaction. CD4+ T cells were isolated from naïve splenocytes using Dynal beads and transfected with STAT4 AS, STAT4 MM, or unrelated oligonucleotides in complex with Lipofectamine. After stimulation with rIL-12 for 48 h the cells were harvested, nuclear proteins were extracted, and a gel retardation assay (EMSA) was performed using a STAT4-specific DNA binding site. D, FITC-labeled STAT4 AS DNA is taken up by splenic T cells. Healthy mice were injected i.v. with FITC-labeled, phosphorothioate-modified STAT4 AS DNA. Eight hours after administration of the drug, mice were killed and cryosections of the spleens were counterstained with anti-CD3. An example of a CD3 and FITC oligonucleotide double-positive T cell is shown, demonstrating successful uptake of the DNA by T cells.

STAT4 AS oligonucleotide treatment suppresses clinical and histopathological signs of arthritis in vivo

In a subsequent series of studies, we aimed at the analysis of the effects of STAT4 oligonucleotides in vivo. We found that the i.v. injection of FITC-labeled STAT4 AS oligonucleotides resulted in an uptake of the DNA by CD3+ splenic T cells (Fig. 4D). This system enabled us to evaluate the in vivo effects of the STAT4 AS oligonucleotides on the clinical and histological outcome in collagen immunized mice (CIA). Based on the kinetics of STAT4 protein expression in splenocytes of mice after CIA induction, we decided to administer the AS reagents on day 26 and to repeat the application on day 33 after immunization. Mice were injected i.v. with STAT4 AS oligonucleotides, STAT4 MM oligonucleotides, or the solvent PBS alone at the same time points. As shown in Fig. 5A, treatment with STAT4 AS oligonucleotides resulted in a significantly delayed onset of arthritis manifestation and in an ~70% reduction of the clinical end score at day 40 as compared with mice injected with STAT4 MM oligonucleotides or just PBS. These results indicate that interference with transcribed STAT4 mRNA by administering STAT4 AS oligonucleotides ameliorates the course of the disease in a specific manner.

In subsequent studies, we performed histopathological comparisons of paraffin sections from STAT4 AS-, STAT4 MM-, and PBS-treated DBA/1 mice. Mice were killed on day 40 after immunization and the hind paws were removed for histological studies. Consistent with the clinical assessment, all histological aspects of arthritis were strikingly reduced after STAT4 AS oligonucleotide treatment as compared with administration of identical amounts of STAT4 MM DNA and PBS (Fig. 5B). Fig. 5C shows representative histopathological evidence for significant reduction of joint inflammation and joint destruction after the administration of STAT4 AS oligonucleotides in comparison with STAT4 MM and PBS treatment. These results showed that, in addition to the reduced clinical manifestation, STAT4 AS oligonucleotide treatment also strikingly diminishes the joint damage in CIA.
Discussion

In the present study, we have identified a crucial role of the transcription factor STAT4 in the pathogenesis of CIA. Furthermore, by using STAT4 AS oligonucleotides this study suggests that the disease-promoting action of STAT4 especially takes place during the manifestation phase of the disease. Our data imply that specific targeting of STAT4 might be a relevant future therapeutic approach in RA treatment.

STAT4 plays a multifaceted role in the immune system, as STAT4 is activated by a number of cytokines (e.g., IL-2, IL-12, IL-15, IL-21, IL-23, and IL-27) (16, 29–31). All of these cytokines are known to promote autoimmune disorders, which highlights STAT4 as an appropriate therapeutic target for the attenuation of disease progression by specifically intervening with its transcriptional effects. Presumably, the best characterized roles for STAT4 are the transcriptional mediation of IL-12-induced production of IFN-γ, the regulation of the IL-12Rβ2 chain expression, and the promotion of the differentiation of naive T cells into proinflammatory Th1 effector cells (15).

Several recent studies suggested a more complex picture of IL-12 action in murine CIA. First, although early Ab mediated neutralization of the IL-12 p40 subunit starting with immunization did not lower the incidence but significantly diminished the severity of arthritis, treatment of mice with established arthritis led to an exacerbation of clinical symptoms (32, 33). These results suggest that IL-12 might play a dual role in CIA. Secondly, IL-12Rβ2−/−, IFN-γ−/−, IFN-γR−/−, and STAT1−/− mice, although lacking critical hallmarks of proinflammatory Th1 T cells, somehow show unexpectedly enhanced susceptibility to various autoimmune disease models (34–37). However, in contrast to IL-12 p35-deficient mice, lacking the p40 subunit showed a dramatically reduced susceptibility and severity of CIA, suggesting that IL-23 p19/p40 may play an important role in CIA (17, 38). Indeed, by using p19-deficient mice it could be demonstrated that, similarly as in EAE (39), arthritis susceptibility is dependent on the presence of the IL-23 p19 subunit (17). Hence, the shared use of the p40 subunit by IL-23 and IL-12 provided a possibility to explain the obvious dichotomy of the p40−/− and p35−/− phenotype in various classical Th1-mediated animal models (17, 19).

Beyond the shared use of the p40 subunit, IL-12 and IL-23 converge on the transcriptional level by activating the same intracellular transcription factor, STAT4. The significance of the STAT4 activation in CIA has not yet been analyzed. In our study we demonstrate that STAT4−/− mice show a dramatically reduced incidence and severity of CIA development. This result provides further significant insight into the IL-12/IL-23/STAT4 balance in CIA for several reasons. First, it proves that STAT4-mediated pathways are crucial for the development of arthritis. These data are consistent with other reports investigating the contribution of STAT4 to different autoimmune disease models such as EAE, experimental autoimmune myocarditis, and proteoglycan-induced arthritis (40–42). Second, the clinical outcome of STAT4−/− mice in CIA is similar to the weak or absent CIA induction in p40−/− mice that lack both IL-23 and IL-12 (38). However, other cytokines such as IL-15 may activate STAT4 signaling and contribute to an arthritis manifestation that would be compromised in STAT4−/− mice (43). Thus, it is possible that cytokine signaling in addition to IL-12 and IL-23 signaling is impaired in STAT4-deficient mice during CIA. However, the question remains as to how disrupted STAT4 signaling suppresses CIA. A defective priming of naive T cells could take place as a consequence of abrogated IL-12 signaling in STAT4−/− mice, leading to a reduced disease manifestation. However, STAT4−/− mice did not show any reduction in the primary proliferative response to Ag in comparison to WT mice, ruling out grossly inhibited priming. Furthermore, there was no evidence for a direct regulatory effect of STAT4 on IL-6 and TNF production during the early phases of CIA, indicating that the reduced IL-6 and TNF levels at late stages
of the disease are secondary to the effects of STAT4 on IFN-γ production.

STAT4 expression in CD4+ T cells from WT mice was clearly up-regulated during CIA in a temporally orchestrated, biphasic manner. STAT4 expression was also up-regulated in CD11b monocytes, consistent with a recent report that monocytes derived from the synovial tissues of RA patients express STAT4 (28). However, the kinetics of STAT4 expression during the onset and early progression of arthritis might be correlated with the known dynamics of the disease-promoting cytokine expression in CIA induced by a specific cytokine milieu. Marinova-Mutafchieva et al. (44) analyzed the cytokine expression pattern of lymph node cells from days 1 to 10 after the onset of arthritis. Interestingly, these cells produced rapidly decreasing amounts of IFN-γ after collagen restimulation, whereas the amounts of IL-6 and TNF were progressively increasing (44). Thus, we suggest that the very distinct and organized expression profile of proinflammatory cytokines released by arthritic T cells is cytokine milieu dependent, e.g., an IL-12- and sequentially IL-23 driven and, at least in part, STAT4-mediated process during the early stages of arthritis that is reflected by a similarly orchestrated STAT4 expression pattern. In any case, STAT4 in T cells was found to regulate the expression of various proinflammatory cytokines, such as IFN-γ, IL-6, TNF, and IL-17, that are known to play a pivotal role in CIA (44).

The specific pattern of STAT4 expression in the course of disease manifestation led us to further analyze the functional role of STAT4 at this stage of arthritis. To address this issue, we developed an AS strategy using oligonucleotides that were directed against the translational start site of STAT4 mRNA. STAT4 AS oligonucleotides led to a significant reduction of STAT4 mRNA levels and the diminished binding to a STAT4 consensus binding site by STAT4 derived from nuclear extracts after AS DNA treatment. Importantly, transient transfection of CD11b+ or CD19+ splenocytes with STAT4 AS or MM oligonucleotides did not alter their cytokine expression profile (IL-1, TNF, and IFN-γ in CD11b+ cells and IL-10 and IFN-γ in CD19+ B cells) under stimulation, arguing against unspecific activating signals elicited by the oligonucleotide sequences used (data not shown). These data encouraged us to use this approach for CIA therapy in vivo. We treated WT mice with CIA by injecting STAT4 AS and MM oligonucleotides i.v. on day 26 after immunization and repeated such treatment 1 wk later. Strikingly, treatment with STAT4 AS DNA but not with MM DNA led to a significant reduction of both clinical and histopathological signs of arthritis. These findings have several implications for the pathogenesis of CIA. The data imply that the biphasic pattern of STAT4 protein expression in CIA is functionally relevant. Based on our results and the work of others (44), we speculate that the STAT4 expression kinetics may precede the expression and mimic the dynamics of the expression of crucial arthritis mediators as IFN-γ, IL-6, and TNF during the onset of disease. Our findings suggest that at this stage of CIA-STAT4 is predominantly a proinflammatory transcriptional mediator whose targeting is beneficial for CIA. In conclusion, the observation of naturally occurring STAT4 expression during arthritis manifestation and successful interference with STAT4 action by an AS approach further support the above findings in knockout studies and suggest that STAT4 plays a pivotal role in CIA. Importantly, STAT4 plays a major role especially at later stages of CIA rather than exclusively regulating early T cell priming and thus emerges as a key novel therapeutic target in chronic autoimmune arthritis.

Taken together, it appears that STAT4 activation in CIA is likely to be mediated by various proinflammatory cytokines, such as IL-12, IL-23, and IL-15, that converge to induce STAT4 activation. Our data suggest a model in which STAT4 drives the production of IFN-γ and thereby Th1 T cell effector function in CIA. STAT4-induced Th1 T cell activation subsequently causes the production of key downstream effector molecules such as IL-6 and TNF, thereby promoting synovial inflammation, tissue destruction, and further recruitment of effector cells. Whether, in later stages of CIA, STAT4 is additionally central for IL-23-mediated expansion of synovial Th17 T cells—a recently identified T cell subset that secretes IL-17, IL-6, and TNF and critically contributes to arthritis manifestation—remains to be analyzed in future studies (20, 21, 45).

Thus, our data unequivocally identify STAT4 as an important transcriptional mediator of proinflammatory signals leading to arthritis manifestation. Specific targeting of STAT4 expression and activation by AS strategies thus emerges as a highly effective approach for the treatment of experimental arthritis with important implications for the design of novel therapies for RA in humans.

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References
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