Alteration of IL-17 Related Protein Expressions in Experimental Autoimmune Myocarditis and Inhibition of IL-17 by IL-10-Ig Fusion Gene Transfer

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Background  T-helper (Th)1/Th2 cytokine balance plays an important role in the pathogenesis of myocarditis. Recently, some studies indicate that interleukin (IL)-17, known as a T cell (Th17)-derived proinflammatory cytokine, is the major mediator of tissue inflammation in inflammatory and autoimmune diseases. Experimental autoimmune myocarditis (EAM) is a T cell-mediated autoimmune disease; however, the pathogenic role of IL-17 in the development of rat EAM remains largely unknown.

Methods and Results  In the present study, alterations of IL-17-related protein expressions were investigated and then the effect of hydrodynamic-based delivery of plasmid DNA encoding the IL-10-Ig gene on rat EAM and the effect of IL-10-Ig on IL-17 was evaluated. The results showed that IL-17 was expressed more highly than IFN-γ expressed by Th1 cells in EAM hearts and the peaks of IL-17 related protein expression in the heart were the early phase of EAM. Moreover, we observed that IL-10-Ig gene therapy was effective in controlling EAM and that IL-10-Ig significantly suppressed the expression of IL-17 as well as other proinflammatory cytokines, IL-10 and TNF-α, in IL-1-stimulated splenocytes cultured from EAM rats.

Conclusions  IL-17 is highly produced by EAM hearts and IL-17 inhibition might be a possible mechanism of the amelioration of EAM by IL-10-Ig treatment. These data suggest that IL-17 produced by Th17 plays an important role in the pathogenesis of rat EAM. (Circ J 2008; 72: 813–819)

Key Words:  Cardiomyopathy; Gene therapy; Interleukin-17; Th17; Th1/Th2 balance

Rat experimental autoimmune myocarditis (EAM) induced by immunization with cardiac myosin is a T cell-mediated autoimmune disease transferred by spleen cells from EAM rats and most infiltrating T cells are CD4⁺ T cells that express CD4⁺ T-helper (Th) cells are traditionally thought to differentiate into interleukin (IL)-2- and IFN-γ-producing Th1 and IL-4+, IFN-γ- and IL-13-producing Th2 cell subsets. It has been assumed that Th1/Th2 cytokine balance is important in myocarditis. Recently, however, Th17 has been reported to play an important role for various models of immune-mediated tissue injury, including organ-specific autoimmunity. Th17 produces IL-17 and it is a proinflammatory cytokine that activates T cells and other immune cells to produce a variety of cytokines, chemokines and cell adhesion molecules. IL-17 cannot be categorized as either Th1 or Th2 cytokine by cytometric single-cell analysis of Th cell cytokine production. Th17 is a new CD4⁺ helper T cell subset characterized by producing IL-17. Th17 cells are activated by IL-23, which is thought to be produced by activated macrophages and dendritic cells via a receptor complex composed of IL-12 receptor (IL-12Rβ1) and IL-23 receptor (IL-23R). IL-17 has been suggested to be involved in the development of rheumatoid arthritis and experimental autoimmune encephalomyelitis (EAE). Recently, it has been reported that mice lacking T-bet, a T-box transcription factor required for Th1 cell differentiation, develop severe autoimmune myocarditis by immunization with myosin heavy chain peptide and anti-IL-17 antibody or neutralization of IL-17 by active vaccination therapy, which markedly reduced mice EAM severity. However, in rat EAM hearts, the time-course of expression of IL-17 related proteins and their producing- or targeting-cells has not been determined.

Th1 T cells producing IL-2 and IFN-γ infiltrate the heart in the early phase of EAM and are estimated to play an important role in triggering EAM. In contrast, Th2 cytokines, IL-4, IL-10 and IL-13 and so on, are thought to play an important role in the control of T-cell-mediated autoimmunity. We have previously reported that IL-10 and IL-13 ameliorated EAM and IL-10 expression increased later than Th1 cytokine in the EAM heart. Recent studies have
shown that both IFN-\(\gamma\) (Th1 cytokine) and IL-4 (Th2 cytokine) negatively regulated the T helper cell production of IL-17 in the effector phase.\(^{20}\) IL-10 is one of the Th2 cytokines, however, the relationship between IL-10 and IL-17 is still unknown in autoimmune disease models. IL-10 in EAM hearts was mainly detected in non-cardiomyocytic non-inflammatory (NCNI) cells (mainly fibroblasts, smooth muscle cells, and endothelial cells)\(^{21,22}\) and IL-10-targeting cells, which expressed both IL-10R1 and IL-10R2, were mainly T cells expressing \(\Delta T\) T cell antigen receptors (\(\Delta T\) cells) and CD11b\(^+\) cells (macrophages/dendritic cells/granulocytes) in EAM hearts.\(^{21,22}\) Several studies have demonstrated the therapeutic effect of IL-10 in various autoimmune/inflammatory diseases. In myocarditis, IL-10 inhibited the secretion of proinflammatory cytokines such as TNF-\(\alpha\), IFN-\(\gamma\), iNOS, IL-2, and IL-12 and displayed major effects on immune cells.\(^{23,24}\)

In this study, we examined the time-course and expression cells of IL-17, IL-17R, IL-23 and IL-23R in EAM hearts, the effect of hydrodynamic-based delivery of the IL-10-Ig fusion gene in rat EAM, and the effect of IL-10 on the expression of IL-17 and proinflammatory cytokines.

**Methods**

*Animals*

Eight-week-old male Lewis rats were purchased from Charles River Laboratories and were maintained at our animal facilities. Throughout the studies, all of the animals were treated in accordance with the guidelines for animal experiments as laid out by our institute (Animal Resources Branch of Niigata University, Niigata, Japan).

*Induction of EAM*

Whole cardiac myosin was prepared from the ventricular muscle of porcine hearts as previously described.\(^ {25}\) It was dissolved in a solution of 0.3 mol/L KCl at a concentration of 10 mg/ml. To produce EAM, on day 0, each rat was immunized with 0.2 ml of an emulsion containing cardiac myosin with an equal volume of complete Freund’s adjuvant (CFA) by a single s.c. injection in both footpads. Rats were immunized with CFA alone as the control group.

*Quantification of IL-17, IL-17R, IL-23, IL-23R, IL-10 and IFN-\(\gamma\) mRNA in EAM Hearts*

To examine the time-course of IL-17, IL-17R, IL-23, IL-23R, IL-10 and IFN-\(\gamma\) expression in EAM, total RNA was isolated from EAM hearts on day 0 (n=3), 6 (n=4), 9 (n=3), 12 (n=3), 15 (n=5) and 18 (n=4) using TRIZol (Invitrogen Life Technologies, Tokyo, Japan), and cDNA was synthesized from 2-5 \(\mu\)g of total RNA with random primers and murine Moloney leukemia virus reverse transcriptase. To create the plasmids used for the standard, IL-17, IL-17R, IL-23, IL-23R, IL-10 and IFN-\(\gamma\) mRNA were amplified from EAM hearts derived cDNA library using the primer pairs for IL-12R \(\alpha\), IL-23R, IL-12R \(\beta\), IL-10 and IFN-\(\gamma\). The lute copy numbers of their mRNA were also measured by quantitative real-time RT-PCR using the primer pairs described above and the primer pairs for IL-12R \(\beta\) (sense primer 5’-ggacttgagctgctacagggtttc-3’, antisense primer 5’-ccaggttacaggtactggrta-3’). These cells were isolated after collagenase perfusion treatment for 20 min using Langendorff apparatus as reported previously.\(^ {21}\) Briefly, isolated cells were separated serially through 30 \(\mu\)m and 20 \(\mu\)m stainless steel sieves twice into cardiomyocytes and other cells respectively. Because almost all inflammatory cells in EAM are \(\Delta T\) T cells and CD11b\(^+\) cells, the other cells were separated into \(\Delta T\) T cells, CD11b\(^+\) cells and NCNI cells such as fibroblasts, smooth muscle cells or endothelial cells by anti-PE micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and a MACS magnetic cell sorting system (Miltenyi Biotech) using appropriate monoclonal antibodies, namely PE-conjugated TCR \(\alpha\)-d (R73) and CD11b (OX-42) (Pharmingen, San Diego, CA, USA). Total RNA was isolated from each purified cell fraction of EAM hearts using TRIZol, cDNA synthesis and quantification of IL-17, IL-17R, IL-23, IL-23R, IL-12R \(\beta\) and IFN-\(\gamma\) mRNA were executed as described above.

*Construction of Plasmid DNA for Gene Transfer*

We prepared the plasmid vector pCAGGS-Ig-glucagon (GLU)-tag containing Swal and NotI restriction sites, the control plasmid, pCAGGS-rat signal peptide (SP)-Ig-GLU-tag, the SP region of the secretory leukocyte protease inhibitor, as previously described.\(^ {26}\) To construct the pCAGGS-rat IL-10-GLU-tag plasmid, rat IL-10 was amplified from EAM heart cDNA using the primers 5’-ttcaTTTTAATgctg-gctgactgctagt-3’ and 5’-gcctgGCGGCCGCTtttttagattgtgtgat-3’, followed by insertion into a pCAGGS-Ig-GLU-tag using Swal and NotI sites. E.coli JM109 competent cells were then transformed, and recombinant plasmids were isolated using a Quantum Prep Plasmid Maxiprep kit (Bio-Rad, Hercules, CA, USA).

*Hydrodynamic-Based Plasmid DNA Encoding IL-10-Ig Gene Injection*

Twenty-four rats were divided into 3 groups (CFA group n=6, IL-10-Ig group n=10, SP-Ig group n=8). CFA group rats were immunized with CFA on day 0. IL-10-Ig and SP-Ig groups rats were immunized with cardiac myosin emulsified in CFA on day 0 and injected with 800 \(\mu\)g of a pCAGGS-rat IL-10-GLU-tag or a pCAGGS-SP-Ig-GLU-tag mixed with the appropriate volume of Ringer’s solution (receiving approximately 80 ml/kg of body weight) via the tail vein within 15 s on day 1, respectively.\(^ {27}\) We previously showed the

\(^{1}\) PCR-amplified cDNA inserts were directly inserted into the pGEM-T easy vector, and recombiant plasmids were isolated following transformation into *Escherichia coli* (*E. coli*) JM109-competent cells using a MagExtractor plasmid kit (Toyobo, Osaka, Japan). Absolute copy numbers of their mRNA were also measured by quantitative real-time RT-PCR using a LightCycler instrument together with the same primers and SYBR Premix Ex Taq (Takara, Otsu, Japan). The absolute copy numbers of particular transcripts were calculated by LightCycler software using a standard curve approach.\(^ {19}\)
plasma chimeric GLU-tag protein measurement

IL-10-Ig because this method was thought to be more effective than in vivo electroporation and chimeras with immunoglobulin-facilitated elevated concentration levels.

Plasma Chimeric GLU-Tag Protein Measurement

To measure plasma concentrations of IL-10-Ig-GLU-tag proteins during the treatment course, blood samples were taken on day 2, 7, 12, and 17 after the hydrodynamics-based gene transfer on day 1. GLU concentrations were measured using a GLU RIA kit (Daiichi Radioisotope Laboratories, Tokyo, Japan). Chimeric protein concentrations were calculated using a GLU-tag.

Evaluation of Histopathology

All rats were killed on day 17. The heart weight without atria and the body weight were measured, and the ratio of heart weight to body weight (g/g) was calculated. The hearts were cut into three 4μm-thick transverse sections for Azan-Mallory staining. The myocarditis areas were determined using specimens stained with Azan-Mallory by a color image analyzer (MacSCOPE, version 2.6; Mitani, Japan).

Expression of ANP mRNA in the Heart

In order to examine the effect of therapy, the amounts of ANP mRNA as a heart failure marker were measured. RNA was extracted from the hearts of rats on day 17 and cDNA was synthesized as described above. ANP mRNA was measured by quantitative real-time RT-PCR using the primer pairs (sense primer S'-atgatttcagacgacgatgac-3', antisense primer 5'-gtgcaactctgcattcatcct-3') and the control was measured using the primer pairs (sense primer S'-cgtcatccagggacgcatc-3', antisense primer 5'-aagggagtgttgggagt-3'). The plasmids used for the standard were constructed as described above.

Preparation of Medium or Serum Containing IL-10-Ig for Spleen Cell Culture

For preparation of medium containing IL-10-Ig-GLU or Ig-GLU, Cos-7 cells (ATCC, Rockville, MD, USA) were cultured on 35 mm-well dishes in 2 ml of RPMI 1640 medium supplemented with 10% FCS for 24 h at 37°C in a humidified atmosphere (5% CO2 and 95% air) and transfected with plasmids using FuGENE-6 (Roche Diagnostics, Indianapolis, IN, USA). Briefly, after reaching confluency, 3μl of FuGENE-6 was incubated in 97μl of serum-free RPMI 1640 medium for 5 min at room temperature and then 1μg of pCAGGS-IL-10-Ig-GLU or sp-Ig-GLU was added, mixed and incubated for 15 min at room temperature. The 100μl of the transfection reagent: DNA was added into each dish and incubated at 37°C in a humidified atmosphere (5% CO2 and 95% air). After 24 h, the medium was changed and at 3 days after the medium was changed, the culture medium was collected and GLU concentrations were measured as described above. The concentration of IL-10-Ig-GLU or Ig-GLU in the medium was 5 nmol/L and we used the medium for spleen cell culture.

Spleen Cell Culture

Spleens were obtained from rats injected with EAM on day 14 and cultured to 6×10^6/ml on 35 mm-well dishes in 2 ml of RPMI 1640 medium supplemented with 10% FCS. Shortly after culture, spleen cells (n=5) were stimulated by adding rat IL-1β (final concentration of 1 ng/ml) (PeproTech, London, UK) and 100μl of IL-10 containing medium.
Statistical Analysis
Statistical assessment was performed by using a non-paired Student’s t-test or a one-way ANOVA and Bonferroni’s multiple comparison test. Differences were considered significant at p<0.05. The heart weight to body weight ratio, myocarditis area, data obtained from quantitative RT-PCR and the concentrations of IL-10-Ig-GLU-tag and Ig-GLU-tag were expressed as the mean ± SEM.

Results
Time-Course of IL-17-Related Proteins mRNA in EAM Hearts
The onset of EAM is approximately 10 days after the injection of a cardiac myosin protein and the peak of inflammation is approximately on day 12 or day 15. mRNA of IL-17 and IL-23R in EAM hearts increased on day 9 and...
mRNA of IL-17, IL-17R, IL-23, IL-23R and IFN-γ peaked on day 12. One of the most remarkable findings was a strong expression of IL-17. Absolute copy numbers of IL-17 mRNA were approximately 30-fold more than those of IFN-γ. In contrast, IL-10 mRNA was augmented on day 18 (Fig 1). The IL-10 expression in EAM hearts was later than for IL-17, IL-23, IL-23R and IFN-γ.

mRNA of IL-17-Related Proteins in Separated Cells From EAM Hearts

In EAM hearts on day 18, IL-17, IL-23R IL-12Rβ1 and IFN-γ were expressed mostly in CD4+ T cells fractions. Similarly to analysis of the time-course, one of the most remarkable findings was a strong expression of IL-17 in CD4+ T cells. Absolute copy numbers of IL-17 mRNA were approximately 20-fold more than those of IFN-γ. IL-17R was detected in CD4+ T cells, CD11b+ and NCNI cell fractions. IL-23 was detected mostly in CD11b+ cell fractions (Fig 2).

Time-Course of Plasma IL-10-Ig-GLU-Tag Protein Levels

During the course of treatment, plasma IL-10-Ig-GLU-tag protein levels in rats injected with pCAGGS-IL-10-Ig on day 1 increased to 1.73±0.25 nmol/L (mean ± SEM) on day 2 and gradually decreased on days 7, 12, and 17 to 0.29±0.03, 0.18±0.03 and 0.07±0.02 nmol/L, respectively. Plasma Ig-GLU-tag protein levels in pCAGGS-SP-Ig control rats increased to 12.5±2.5 nmol/L on day 2 and decreased on days 7, 12 and 17, to 9.74±2.86, 8.99±2.52 and 2.36±1.06 nmol/L, respectively (Fig 3a). Although IL-10-Ig-GLU-tag serum levels were lower than that of control Ig-GLU-tag serum levels by hydrodynamic-based gene delivery, they were higher than the serum level found by in vivo electroporation gene transfer into a muscle.17 These results indicated that a continuous effective delivery of the IL-10-Ig-GLU-tag protein could be achieved in rats by hydrodynamic-based gene transfection.

Effect of In Vivo Treatment With Plasmid DNA Encoding the IL-10-Ig Gene

The heart weight to body weight ratio of the IL-10-Ig group was significantly less than that of the SP-Ig group (0.49±0.02 vs 0.54±0.02, p=0.0172) (mean ± SEM) (Fig 3b). The inflammatory area of the ventricle transverse section in the IL-10-Ig group was significantly smaller than that in the negative control group (0.49±0.02 vs 0.54±0.02, p=0.0172) (mean ± SEM) (Fig 3b).

Fig 4. A histological examination of transverse sections in ventricles were stained with Azan-Mallory stain. (a) A transverse section of a heart in a complete Freund’s adjuvant control group did not exhibit myocarditis. (b) Transverse sections of hearts in the SP-Ig-GLU-tag group. (c) Transverse sections of hearts in the IL-10-Ig-GLU-tag group. Bar represents 1 mm.

Fig 5. Copy numbers of interleukin (IL)-17 (a), IL-1 (b), TNF-α (c) and IFN-γ (d) mRNA per copy number of β-actin mRNA in cultured spleen cells (n=5) from experimental autoimmune myocarditis rats. Negative control, none of IL-1, medium and serum; IL-1+ SP medium, IL-1 (1 ng/ml) and 5% culture medium obtained from Cos-7 cells transfected with plasmid pCAGGS-SP-Ig-GLU-tag; IL-1+ IL-10 medium, IL-1 (1 ng/ml) and 5% culture medium obtained from Cos-7 cells transfected with plasmid pCAGGS-IL-10-Ig-GLU-tag; IL-1+ SP serum, IL-1 (1 ng/ml) and 5% serum obtained from a normal rat injected with pCAGGS-SP-Ig-GLU-tag; IL-1+ IL-10 serum, IL-1 (1 ng/ml) and 5% serum obtained from a normal rat injected with pCAGGS-IL-10-Ig-GLU-tag. The final concentration of the IL-10-Ig protein or the SP-Ig protein in the culture dish was 0.25 nmol/L. Error bars represent SEM. Statistical assessment was performed by using a one-way ANOVA and Bonferroni’s multiple comparison test (negative control vs IL-1+SP medium vs IL-1+IL-10 medium or a negative control vs IL-1+SP serum vs IL-1+IL-10 serum). Results are representative of 3 independent experiments.

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controls (21.3±3.2 vs 42.7±2.4, p=0.0001) (Fig 3c). Inflammation and fibrosis in the hearts of the SP-Ig-GLU-tag group was more severe than that of the IL-10-Ig group. The ratio of ANP mRNA to Cox6a2 as a heart failure marker in the IL-10-Ig group was significantly lower than that in controls (8.5±2.4 vs 24.6±7.3, p=0.0155) (Fig 3d). The hearts from immunized CFA control rats did not show inflammation (Fig 4).

mRNA Expression of IL-17 and Immunologic Molecules in Cultivated Spleen Cells After IL-10 Treatment

We examined the effect of IL-10 on the expression of IL-17 in cultivated spleen cells (n=5) from EAM rats on day 14. The concentration of the IL-10-Ig protein (final concentration of 0.25 nmol/L) used in the in vitro experiment is almost the same as that of serum in the early phase of EAM, when it was treated with a hydrodynamic-based IL-10-Ig gene transfer. The results showed that IL-17 was significantly enhanced by IL-10 and IL-10-Ig-containing medium and that serum significantly inhibited the gene expression of IL-17 in IL-1-stimulated spleen cells from EAM rats (Fig 5). We also examined the effect of IL-10 on the expression of other proinflammatory cytokines such as IL-1, TNF-α, and IFN-γ in IL-1-stimulated spleen cells from EAM rats, and the results showed that IL-10-Ig-containing medium or serum significantly inhibited the gene expressions of IL-1, TNF-α, and IFN-γ, however, IFN-γ was not significantly inhibited by IL-10-Ig-containing medium or serum (Fig 5).

Discussion

IL-17 was expressed in the early phase of EAM. The time-course of the expression of IFN-γ was similar to that of IL-17 in EAM hearts. Because EAM is a T-cell-mediated disease and most of the infiltrating T cells in EAM are CD4+ Th17 cells producing IL-17 might trigger or sustain inflammatory events in the heart. Until recently, we have suggested that Th1 cells producing IFN-γ or IL-2 played an important role as a trigger of EAM; however, in this study, our results indicate that absolute copy numbers of IL-17 mRNA are approximately 20-30-fold more than those of IFN-γ mRNA in EAM hearts. This is consistent with results involving IFN-γ from previous studies and absolute copy numbers of IL-17 mRNA measured in this study are approximately 300-fold more than those of IL-2 mRNA obtained from previous data. It has been reported that IL-17-producing CD4+T cells in CNS lesions of EAE behaved similarly to IFN-γ-producing cells but IL-17-producing cells outnumbered IFN-γ-producing cells and that neutralization of IL-17 with a monoclonal antibody ameliorated disease. Steinman has recently reviewed the roles of Th cells subsets. He proposes that Th1 cells, long thought to mediate tissue damage, might be involved in the initiation of damage but that they do not sustain or play a decisive role in many commonly studied models of inflammation and that a pathway named Th17 is now credited for causing and sustaining tissue damage. IL-23, which is suggested to be responsible for the differentiation and expansion of Th17 cells from naive CD4+T cells was also expressed in the early phase of EAM and in CD11b+ cells. IL-23 produced by CD11b+ cells, macrophages or dendritic cells and so on might promote IL-17 production in Th17 cells and trigger inflammatory events in hearts.

In this study, it is interesting that IL-10-Ig-containing medium or serum significantly inhibited the gene expression of IL-17 in IL-1-stimulated spleen cells from EAM rats. IL-10 was previously reported to inhibit proinflammatory cytokines such as IL-1, TNF-α, and IFN-γ; however, the effects of IL-10 on IL-17 remain unknown. Lubberts et al reported that IL-4 gene therapy for collagen arthritis suppressed synovial IL-17 and improved arthritis. In this study, the IL-10-Ig protein suppressed IL-17 gene expression in IL-1-stimulated spleen cells from EAM rats and improved EAM. This might be similar to their report of the IL-4 effect on IL-17. Recently, it was also reported that IL-23 and not IL-12 was essential for the manifestation of chronic intestinal inflammation by producing the proinflammatory mediators IL-17 and IL-6 in IL-10-deficient mice, which spontaneously developed enterocolitis. IL-10 expression in EAM hearts increased later than IL-17, and the recovery of EAM was concomitant with the increase of IL-10 as well as EAE. Sutton et al reported that IL-23-induced IL-17 production was substantially enhanced by IL-1, and IL-1 functional upstream of IL-17 to promote pathogenic Th17 cells in EAE. Both IL-1 and IL-23 are strongly expressed in CD11b+ cells in the early phase of EAM, and IFN-γ cells are thought to be strongly stimulated by IL-1 and IL-23; therefore, IFN-γ cells in EAM are thought to express IL-17 strongly. Our data suggests that IL-10 significantly inhibited IL-17 expression in IFN-γ cells under such conditions. Future research needs to consider why IL-10 inhibits IL-17 expression in IL-1-stimulated spleen cells.

In this study, we investigated the alterations of IL-17- and IL-23-producing and -targeting cells in EAM hearts. We demonstrated that IL-10-Ig treatment by a hydrodynamic-based delivery ameliorated EAM, and that IL-10-Ig significantly inhibited IL-17-producing and -targeting cells in EAM hearts. We also examined the effect of IL-10 on IL-17-stimulated spleen cells from EAM rats. IL-17 was expressed more highly than Th1 cytokines as IFN-γ or IL-2 in IFN-γ cells in the early phase of EAM hearts. These data suggest that IL-10 significantly inhibited IL-17 expression in IFN-γ cells under such conditions. Future research needs to consider why IL-10 inhibits IL-17 expression in IL-1-stimulated spleen cells.

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References

IL-17 Related Protein Expressions in EAM


