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Hydrodynamic-Based Delivery of an Interleukin-22-Ig Fusion Gene Ameliorates Experimental Autoimmune Myocarditis in Rats¹

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IL-22 is one of several cytokines with limited homology to IL-10. However, the biological activities of IL-22 are mostly unknown. The purpose of this study was to evaluate the effect of IL-22 on rat experimental autoimmune myocarditis (EAM) and elucidate an aspect of the biological activities of IL-22. Rats were immunized on day 0; IL-22-Ig-treated rats were injected with pCAGGS-IL-22-Ig and control rats with pCAGGS-Ig using hydrodynamics-based gene delivery on day 1 or day 6. IL-22-Ig gene therapy administered on day 1 or day 6 after immunization was effective in controlling EAM as monitored by the heart weight to body weight ratio, and the myocarditis area in rats was sacrificed on day 17. Examination of the expression of IL-22-related genes in purified cells from EAM hearts suggested that IL-22-Ig acting target cells were noncardiomyocytic (NC) noninflammatory cells such as fibroblasts, smooth muscle cells, and endothelial cells. Therefore, we examined the effect of rIL-22 or serum containing IL-22-Ig on the expression of immune-relevant genes in IL-1-stimulated NC cells cultured from EAM hearts. Results showed that the expression of immunologic molecules (PGE synthase, cyclooxygenase-2, MIP-2, MCP-1, IL-6, and cytokine-induced neutrophil chemoattractant-2) in IL-1-stimulated NC cells was significantly decreased by rIL-22 or serum containing IL-22-Ig. EAM was suppressed by hydrodynamics-based delivery of plasmid DNA encoding IL-22-Ig, and the reason for this effectiveness may be that IL-22 suppressed gene expression of PG synthases, IL-6, and chemokines in activated NC noninflammatory cells. *The Journal of Immunology*, 2006, 177: 3635–3643.

Interleukin-22 belongs to a family of cytokines with limited homology to IL-10, namely IL-19, IL-20, IL-22, IL-24, and IL-26 (1). The cDNA for the mouse IL-22 ortholog was identified as a gene specifically induced by IL-9 in mouse T cells, and the protein was originally designated IL-10-related T cell-derived inducible factor. IL-22, with 22% identity to IL-10, consists of 179 amino acids (2). IL-22 has the potential to interact with IL-10 because it binds to the IL-10RB (IL-10R β) chain with the IL-22RA1 (IL-22R α 1) chain in its receptor complex (3). Both cytokines, IL-10 and IL-22, activate a similar combination of Stat proteins, namely Stat1, Stat3, and Stat5 (3). However, the biological activities of IL-22 are largely unknown and are thought to be quite different from those of IL-10. IL-22 does not inhibit the production of proinflammatory cytokines such as TNF- α or IL-1 by monocytes in response to LPS, nor does it impact IL-10 function on monocytes (4).

Previously, we demonstrated that IL-10 ameliorated rat experimental autoimmune myocarditis (EAM)³ (5). EAM has been shown to be a T cell-mediated autoimmune myocarditis (6). Histopathological investigation showed that CD11b⁺ cells (macrophages, dendritic cells, and granulocytes) and CD4⁺ T cells infiltrated the heart (7). We supposed that the beneficial effects of IL-10 in EAM were due to the inhibition of proinflammatory cytokines produced by these infiltrating cells. In this study we investigated whether IL-22, which was thought not to inhibit this cytokine production by monocytes, suppresses EAM, and we aimed to elucidate an aspect of the biological activities of IL-22.

Materials and Methods

Animals

Eight-week-old male Lewis rats were purchased from Charles River Laboratories and maintained in 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 (8). 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 supplemented

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³ Abbreviations used in this paper: EAM, experimental autoimmune myocarditis; CINC-2, cytokine-induced neutrophil chemoattractant-2; COX-2, cyclooxygenase-2; GLU, glucagon; NC, noncardiomyocytic; NCNI, NC noninflammatory; PGES, PGE synthase; SP, signal peptide; WBC, white blood cell.

with 10 mg/ml *Mycobacterium tuberculosis* H37RA (Difco) by a single s.c. injection in both footpads.

Construction of plasmid DNA for gene transfer

We prepared the plasmid vector pCAGGS-Ig-glucagon (GLU)-tag containing *SwaI* and *NotI* restriction sites, the control plasmid, pCAGGS-rat signal peptide (SP)-Ig-GLU-tag as previously described (9), and the plasmid vector pCAGGS-GLU-tag without Ig containing *SwaI* and *NotI* restriction sites by inserting PCR products amplified with the primers 5'-gaGAATTCATTTAAATgagaCGGCGCCcccaggtaaagcccaagatttggcagtggtg-3' and 5'-gagagagaGAATTCcaggtattcatcaaccactgcacaaaatcttgggc-3' using *EcoRI* sites (capital letters denote restriction endonuclease recognition sequences). To construct the pCAGGS-rat IL-22-Ig-GLU-tag and pCAGGS-rat IL-22-GLU-tag, rat IL-22 was amplified from the cDNA of rat splenocytes stimulated by Con A and rat IL-1 α (PeproTech) using the primers 5'-ttcATTTAAATggctgtcctgcagaatctatg-3' and 5'gcatcGCGGC CGCgagcgaagcattctcagagacat-3', followed by insertion into pCAGGS-Ig-GLU-tag or pCAGGS-GLU-tag using *SwaI* and *NotI* sites. *Escherichia coli* JM109-competent cells were then transformed, and recombinant plasmids were isolated using a Quantum Prep Plasmid Maxiprep kit (Bio-Rad).

In vivo treatment of EAM with the plasmid DNA encoding IL-22-Ig gene

Thirty-five rats were divided into five groups (control group ($n = 7$) with no immunization and no injection; IL-22-Ig group ($n = 6$) with immunization on day 0 and injection on day 1; SP-Ig group ($n = 7$) with immunization on day 0 and injection on day 1; IL-22-Ig group ($n = 9$) with immunization on day 0 and injection on day 6; and SP-Ig group ($n = 6$) with immunization on day 0 and injection on day 6), and immunized rats were injected with 800 μ g of pCAGGS-rat IL-22-Ig-GLU-tag or pCAGGS-SP-Ig-GLU-tag that was mixed with the appropriate volume of Ringer's solution (receiving ~ 80 milliliters per kilogram of body weight) via the tail vein within 15 s on day 1 or day 6 (10).

Plasma chimeric GLU-tag protein measurement and white blood cell (WBC) count

For evaluating the therapeutic benefits of chimeric proteins containing Ig, we examined plasma concentrations of IL-22-GLU-tag or IL-22-Ig-GLU-tag proteins expressed by hydrodynamics-based delivery of pCAGGS-rat IL-22-GLU-tag or pCAGGS-rat IL-22-Ig-GLU-tag in normal rats. Subsequently, for measuring plasma concentrations of IL-22-Ig-GLU-tag proteins during the course of treatment, blood samples were taken on days 2, 7, 12, and 17 after gene transfer on day 1 and on days 7, 12 and 17 after gene transfer on day 6. GLU concentrations were measured using a GLU RIA kit (Daiichi Radioisotope Laboratories) (11). Chimeric protein concentrations were calculated using a GLU-tag, (chimeric protein concentration (nmol/L) = actually measured GLU concentration (ng/L)/whole GLU m.w.) (12). Furthermore, for evaluating the systemic nature of the IL-22 treatment, we measured the WBC counts in control and treated rats. After hydrodynamics-based gene transfer of pCAGGS-rat IL-22-GLU-tag or pCAGGS-SP-Ig-GLU-tag on day 6, blood samples were taken on days 7, 12, and 17. WBC counts were measured using a Sysmex F-820 blood cell counter.

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. Ten percent formalin-fixed and paraffin-embedded hearts

were cut into three 4- μ m-thick transverse sections for H&E and Azan-Mallory staining. The area of the entire heart and the regions affected by myocarditis (regions showing myocardial necrosis, inflammatory cell infiltration, and myocardial fibrosis) were determined using the specimen stained with Azan-Mallory stain by a color image analyzer (MacSCOPE, version 2.6; Mitani). The ratio of the affected area to the entire heart area was calculated.

Immunohistochemical staining of inflammatory cells

For analysis of changes in inflammatory cells infiltrating into the hearts as a result of IL-22 treatment, hearts harvested on day 17 after the gene transfer on day 6 were embedded in Tissue-Tek OCT compound and frozen at -80°C . Six-micrometer-thick transverse sections were cut from the mid-ventricle with a cryostat and fixed in ether for 5 min. Sections were washed in 0.01M PBS, placed into 0.3% H_2O_2 methanol for 5 min, and blocked with 20% normal goat serum for 20 min. The presence of macrophages, CD4^+ T cells, and CD8^+ T cells was examined immunohistochemically using a mouse mAb against rat macrophages (ED1; Serotec), a mouse mAb against rat CD4 (W3/25; Serotec) or CD8 (OX-8; Serotec), biotinylated goat anti-mouse IgG1 (Amersham Biosciences), and labeled streptavidin-HRP (Amersham Biosciences). Total ED1^+ , CD4^+ , and CD8^+ cells of the IL-22 group ($n = 9$) and Ig control group ($n = 6$) were counted, and the ratio of the number of positive cells of each type to the total number of positive cells was calculated.

RNA extraction from cells separated from EAM heart

To evaluate target cells of IL-22 and the intercellular cross-talk by IL-22, the expression of IL-22-related genes was examined in cardiomyocytes, $\alpha\beta$ T cells, CD11b^+ cells, and noncardiomyocytic (NC) noninflammatory (NCNI) cells (mainly fibroblasts, smooth muscle cells, and endothelial cells) in the hearts of EAM rats on day 18 by quantitative real-time RT-PCR. These cells were isolated after collagenase perfusion treatment for 20 min using a Langendorff apparatus as reported previously (13). The fractions of cardiomyocyte, $\alpha\beta$ T cells, CD11b^+ cells, and NCNI cells were confirmed by analysis of the expression of specific marker genes, namely, α -cardiac myosin, CD3 , CD11b , collagen type III, calponin, and von Willebrand factor; even if the level of contamination was the highest, it was $<10\%$ (13). Total RNA was isolated from each purified cell fraction (cardiomyocytes, CD11b^+ cells, $\alpha\beta$ T cells, and NCNI cells) of EAM hearts using TRIzol (Invitrogen Life Technologies), and cDNA was synthesized from 2–5 μ g of total RNA with random primers and murine Moloney leukemia virus reverse transcriptase in a final volume of 20 μ l. Moreover, the absolute copy numbers of PGE synthase (PGES), cyclooxygenase-2 (COX-2), IL-6, cytokine-induced neutrophil chemoattractant-2 (CINC-2), and MIP-2 mRNA were also measured by quantitative real-time RT-PCR.

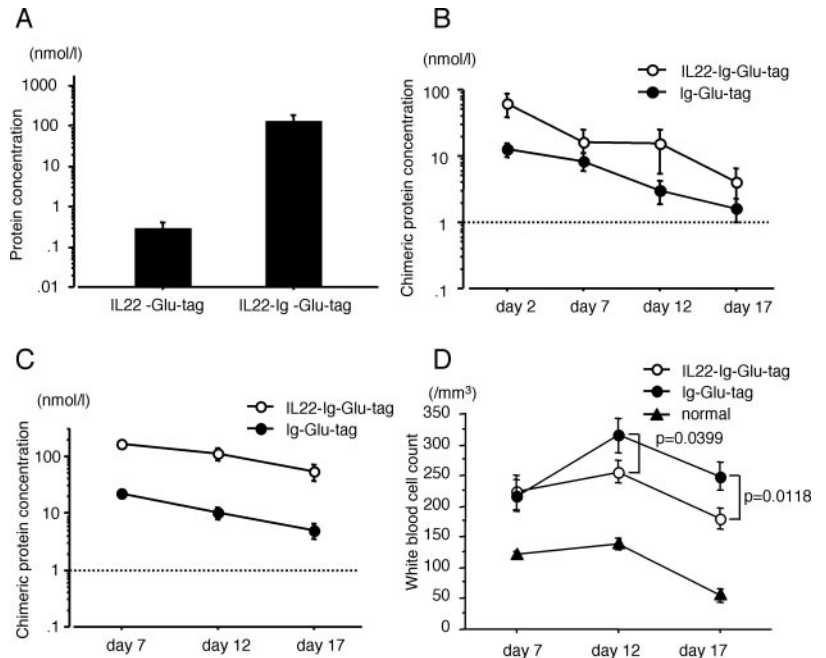
Noncardiomyocytic cells culture with rIL-22 or serum containing IL-22-Ig

On day 18, NC cells were isolated from the hearts of EAM rats via collagenase preparation and cultured for 2 wk on 35-mm-well dishes in 2 ml of RPMI 1640 medium supplemented with 10% FCS. After reaching confluency, NC cells were stimulated by adding 1 ng/ml IL-1 α , 10 ng/ml rat rIL-22 (R&D Systems), or 100 μ l of IL-22-Ig-GLU-tag-containing serum obtained from a IL-22-Ig treated EAM rat on day 17 (6 nmol/L \approx 260 ng/ml) or the same amount of Ig-GLU-tag-containing serum from a SP-Ig treated EAM rat on day 17. After culturing for 24 h at 37°C , NC cells were collected, total RNA was isolated, and cDNA was synthesized as described above. The absolute copy numbers of γ -actin, PGES, COX-2, IL-6,

Table I. List of primers for quantitative RT-PCR

	Sense primer	Antisense primer
IL-22	5'-gataaaaaaacacacagatgtcaggctc-3'	5'-gatcgccttaatctctcactctc-3'
IL-22RAI	5'-gtcactaagatgactgatcgttc-3'	5'-aggttcatttgtagtggtt-3'
IL-10RB	5'-gcgactctcgttctttctaaat-3'	5'-aaactctcttagtcccgctttt-3'
IL-22BP	5'-acagggacaatgaaagataaaaa-3'	5'-aattgttgattatgaaactcgtt-3'
COX-2	5'-tgtgatattctcaaacaggagcat-3'	5'-aaggaggatggagttgttagag-3'
PGES	5'-gtgatggagaacaccaggt-3'	5'-gaggaccacgaggaatgtatc-3'
IL-6	5'-ccgagttagacctatagtgacctt-3'	5'-cctattgaaatctgctctgctt-3'
MCP-1	5'-ctgtctcagccagatgcagttat-3'	5'-tatgggcaagtccattcaaaag-3'
CINC2	5'-cactgcttctgctcttctg-3'	5'-cttgagggttgagacaactctt-3'
MIP-2	5'-agctcctcaatgctgactggt-3'	5'-gttagccttgcctttgttcagat-3'

FIGURE 1. A, Plasma IL-22-GLU-tag protein and IL-22-Ig-GLU-tag protein levels. The normal rats were injected with pCAGGS-rat IL-22-GLU-tag or pCAGGS-rat IL-22-Ig-GLU-tag, and after 24 h each plasma sample was obtained. B, Plasma Ig-GLU-tag protein and IL-22-Ig-GLU-tag protein levels. The rats were injected with pCAGGS-rat SP-Ig-GLU-tag or pCAGGS-rat IL-22-Ig-GLU-tag on day 1. Chimeric protein concentrations were calculated by using the GLU-tag. C, Plasma Ig-GLU-tag protein and IL-22-Ig-GLU-tag protein levels. The rats were injected with pCAGGS-rat SP-Ig-GLU-tag or pCAGGS-rat IL-22-Ig-GLU-tag on day 6. D, WBC count. The symbol marked "normal" represents rats that were neither immunized with cardiac myosin nor injected with a plasmid. The symbol for the IL-22-Ig-GLU-tag represents the rats that were immunized on day 0 and injected with pCAGGS-rat IL-22-Ig-GLU-tag on day 6. The symbol for the Ig-GLU-tag represents the rats that were immunized on day 0 and injected with pCAGGS-rat SP-Ig-GLU-tag on day 6. Statistical assessment was performed by one-way ANOVA and Bonferroni's multiple comparison test. Significant differences between the IL-22-Ig-GLU-tag group and the Ig-GLU-tag group are shown. Error bars represent SEM.



CINC-2, MIP-2, and MCP-1 mRNA were measured by quantitative real-time RT-PCR. These analyses were performed three times to confirm the results. Moreover, the culture medium was collected for ELISA, and IL-6, CINC-2 α , and MCP-1 concentrations were measured using a rat IL-6 immunoassay kit (R&D Systems), a rat CINC-2 α immunoassay kit (IBL), and rat MCP-1 immunoassay kit (BioSource International).

Immunohistochemical staining of cultured cells

For determining the actual ratio of each cell type in cultured cells, NC cells isolated from the heart of EAM rats using the same procedures were cultured for 2 wk on sterile Lab-Tek II chamber slides (Nalge Nunc International) and frozen at -20°C . The slides were immunostained with mouse monoclonal α -smooth muscle actin Abs (Sigma-Aldrich), rabbit anti-factor VIII-related Ag Abs (Zymed Laboratories), rabbit anti-rat collagen type III Abs (Monosan), mouse anti-rat CD11b Abs (OX-42), and mouse anti-rat CD3 Abs (G4.18) (BD Pharmingen). The slides were washed in 0.05M TBS three times. Immunodetection was performed using biotinylated anti-rabbit and anti-mouse Igs followed by alkaline phosphatase-conjugated

streptavidin and fast red chromogen (LSAB2 kit; DakoCytomation) for red staining. The sections were lightly counterstained with Mayer's hematoxylin. Negative control slides were incubated with either mouse IgG2a or normal rabbit serum instead of the primary Ab. The numbers of each positive cell in a 1-mm 2 section were counted, and the ratio of the number of each type of positive cell to the number of total positive cells was calculated.

Quantitative real-time RT-PCR analysis

To create the plasmids used for the standard, the cDNAs for specific cell markers, IL22 family proteins, and immunologic molecules were amplified from an EAM heart-derived cDNA library using the primers indicated in Table I or as previously reported (14). PCR-amplified cDNA inserts were directly inserted into the pGEM-T easy vector, and the recombinant plasmids were isolated following transformation into *E. coli* JM109-competent cells using the MagExtractor plasmid kit (Toyobo). Diluted plasmid and cDNA were amplified via real-time RT-PCR using a LightCycler instrument together with the same primer pair used for making the plasmid and

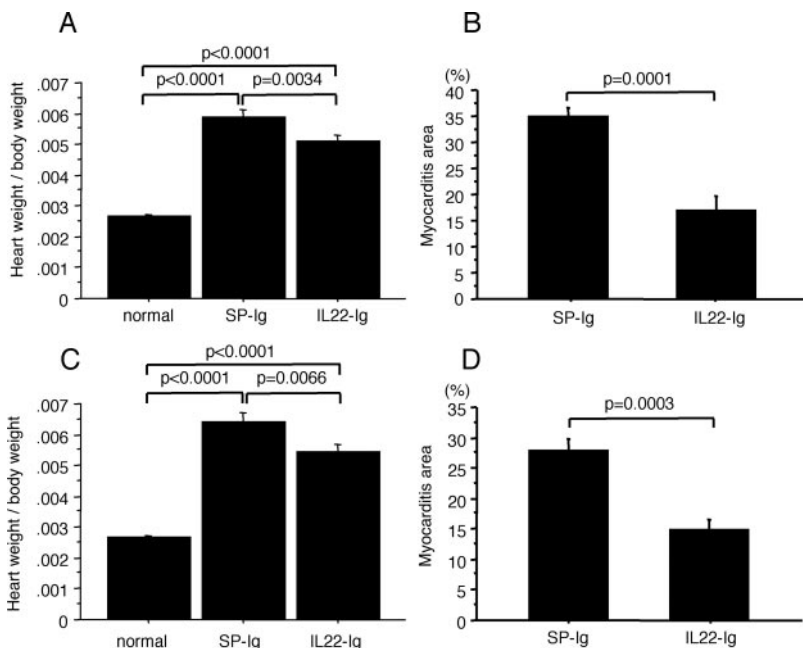


FIGURE 2. A and C, Heart weight to body weight ratio. B and D, Myocarditis area in EAM heart. The area was calculated by a color image analyzer using specimens stained with Azan-Mallory stain. A and B, Rats that were injected with plasmid on day 1. C and D, rats that were injected with plasmid on day 6. The rats were injected with pCAGGS-rat SP-Ig-GLU-tag (SP-Ig) or pCAGGS-rat IL-22-Ig-GLU-tag (IL-22-Ig). Columns marked "normal" represents rats that were neither immunized with cardiac myosin nor injected with plasmid. Error bars represent SEM. Statistical assessment was performed by one-way ANOVA and Bonferroni's multiple comparison test (A and C) or a nonpaired *t* test (B and D).

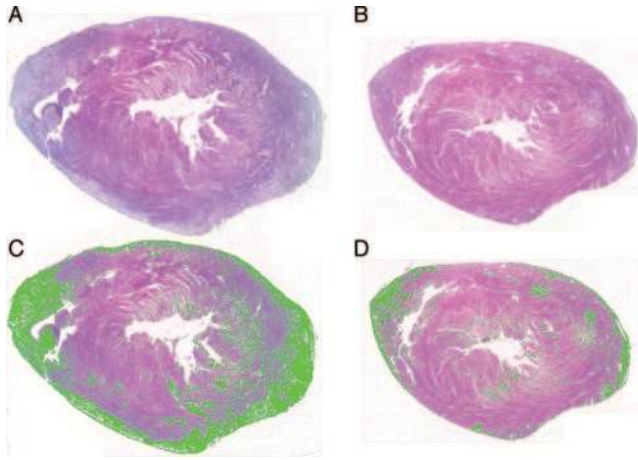


FIGURE 3. Histological examination of transverse sections in both ventricles of rats injected on day 6 were stained with Azan-Mallory stain. *A* and *C*, Transverse sections of hearts in the SP-Ig group. *B* and *D*, Transverse sections of hearts in the IL-22-Ig group. The area was calculated by a color image analyzer using specimens stained with Azan-Mallory stain. Data are representative of each group.

SYBR Premix Ex Taq (Takara Shuzo). After an initial denaturation step for 10 min at 95°C, a three-step cycling procedure (denaturation at 95°C for 10 s, annealing at 62°C for 10 s, and extension at 72°C for 13 s) was used for 45 cycles. The absolute copy numbers of particular transcripts were calculated by LightCycler software using a standard curve approach (15). cDNA from cultivated cells was then subjected to quantitative RT-PCR analysis with the level of γ -actin mRNA acting as the internal control.

Statistical analysis

Statistical assessment was performed by a nonpaired Student *t* test or one-way ANOVA and Bonferroni's multiple comparison test. The differences were considered significant at $p < 0.05$. The heart weight to body weight ratio, the myocarditis area, the data obtained from quantitative RT-PCR ELISA, and the concentration of IL-22-Ig-GLU-tag and Ig-GLU-tag were expressed as mean \pm SEM.

Results

Plasma IL-22-Ig-GLU-tag protein levels and WBC counts

Plasma concentrations of IL-22-Ig-GLU-tag proteins expressed by hydrodynamics-based delivery of pCAGGS IL-22-Ig-GLU-tag are \sim 500-fold higher than those of IL-22-GLU-tag proteins by pCAGGS IL-22-GLU-tag (125 ± 46 nmol/L vs 0.275 ± 0.127 nmol/L) (Fig. 1A). Therefore, we used a chimeric protein containing Ig for EAM treatment. During the course of treatment, plasma IL-22-Ig-GLU-tag protein levels in rats injected with pCAGGS-IL-22-Ig on day 1 increased to 61.7 ± 23.6 nmol/L (mean \pm SEM) on day 2 and gradually decreased on days 7, 12, and 17 to 16.4 ± 7.9 , 15.3 ± 10.3 , and 4.1 ± 2.4 nmol/L, respectively. In contrast, the plasma Ig-GLU-tag protein levels in the pCAGGS-SP-Ig control rats increased to

12.6 ± 3.1 nmol/L on day 2 and decreased on days 7, 12, 17, to 8.4 ± 2.5 , 3.0 ± 1.1 , and 1.6 ± 0.6 nmol/L, respectively (Fig. 1B). Plasma IL-22-Ig-GLU-tag protein levels in rats injected pCAGGS-IL-22-Ig on day 6 increased to 164 ± 16 nmol/L (mean \pm SEM) on day 7 and gradually decreased on days 12 and 17 to 111 ± 27 and 53.4 ± 18.1 nmol/L, respectively. In contrast, the plasma Ig-GLU-tag protein levels in the pCAGGS-SP-Ig control rats increased to 22.3 ± 2.9 nmol/L on day 7 and decreased on days 12 and 17 to 10.2 ± 2.7 and 5.0 ± 1.5 nmol/L, respectively (Fig. 1C). It has been reported that IL-22 (100 ng/ml \approx 5 nmol/L) led to STAT activation (16). These results indicated that a continuous effective delivery of IL-22-Ig-GLU-tag protein until day 17 could be achieved in rats by hydrodynamic-based transfection. WBC counts on days 7, 12, and 17 in rats immunized on day 0 and injected with pCAGGS-SP-Ig or pCAGGS-IL-22-Ig on day 6 were significantly more than those of normal rats. WBC counts levels on day 12 and 17 in IL-22-Ig group were significantly less than those of the SP-Ig group (Fig. 1D). This result shows that IL-22 may influence leukocyte infiltration into the heart by indirectly influencing mobilization of WBCs or hematopoiesis.

Effect of in vivo treatment with plasmid DNA encoding the IL-22-Ig gene

The heart weight to body weight ratio of the IL-22-Ig group was significantly less than that of the SP-Ig group (injection on day 1, 0.51 ± 0.02 vs $0.59 \pm 0.02\%$, $p = 0.0034$; injection on day 6, 0.54 ± 0.03 vs $0.64 \pm 0.03\%$, $p = 0.0066$) (mean \pm SEM) (Fig. 2, *A* and *C*). Many inflammatory cells and fibroblasts had infiltrated SP-Ig group hearts, but fewer inflammatory cells were found in the hearts of the IL-22-Ig treated group. The inflammatory area of the ventricle transverse section in the IL-22-Ig group was significantly smaller than that in the controls (injection on day 1, 16.6 ± 2.9 vs $34.8 \pm 1.6\%$, $p = 0.0001$; injection on day 6, 14.6 ± 1.7 vs $27.7 \pm 2.0\%$, $p = 0.0003$) (Fig. 2, *B* and *D*, and Fig. 3). Analysis of infiltrating cells in EAM hearts showed that the ratio of ED1⁺ cells to total infiltrating cells relatively decreased and the ratio of CD4⁺ and CD8⁺ cells to total infiltrating cells relatively increased with IL-22-Ig treatment significantly (Fig. 4). These results suggest that IL-22 treatment suppresses the infiltration of ED1⁺ cells rather than that of CD4⁺ or CD8⁺ cells.

mRNA of IL-22 related proteins and immunological proteins in separated cells from EAM hearts

mRNA of IL-22-related proteins in cardiac myocyte fractions ($n = 5$), $\alpha\beta$ T cell fractions ($n = 5$), CD11b⁺ cell fractions ($n = 5$), and NCNI cell fractions such as fibroblasts, smooth muscle cells, and endothelial cells ($n = 6$) purified from EAM hearts on day 18 were analyzed by real time RT-PCR. NCNI cell fractions clearly expressed IL-22RA1 mRNA in quantitative real-time PCR analysis. However, IL-22RA1 mRNA levels expressed in $\alpha\beta$ T cells,

FIGURE 4. Infiltrating cells analyzed by immunohistochemistry in EAM hearts from SP-Ig group ($n = 6$) and IL-22-Ig group ($n = 9$). Columns marked SP-Ig represent rats that were injected with pCAGGS-rat SP-Ig-GLU-tag on day 6. Columns marked IL22-Ig represents rats that were injected with pCAGGS-rat IL-22-Ig-GLU-tag on day 6. ED1⁺ cells, CD4⁺ cells, and CD8⁺ cells in transverse heart sections were counted. *A*, ED1⁺ cells per total cells consisting of ED1⁺ cells, CD4⁺ cells, and CD8⁺ cells. *B*, CD4⁺ cells per total cells. *C*, CD8⁺ cells per total cells. Error bars represent SEM. Statistical assessment was performed by a nonpaired *t* test.

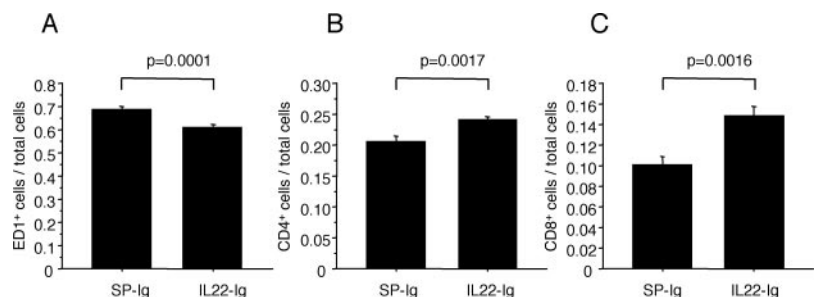
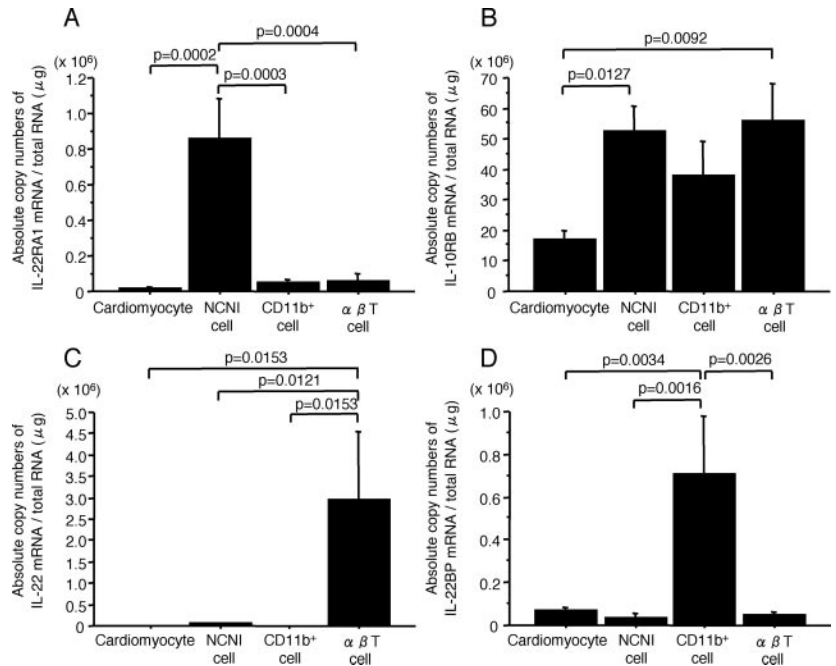


FIGURE 5. Absolute copy numbers of IL-22RA1 (A), IL-10RB (B), IL-22 (C), and IL-22BP (D) mRNA. Each cell fraction ($n = 5$ or 6) was separated and purified from an EAM heart on day 18. NCNI cells are mainly fibroblasts, smooth muscle cells, and endothelial cells. Error bars represent SEM. Statistical assessment was performed by one-way ANOVA and Bonferroni's multiple comparison test. Differences were considered significant at $p < 0.05$.



CD11b⁺ cells, and cardiomyocyte fractions were low (Fig. 5A). IL-10RB, which formed a heterodimer with IL-22RA1, was detected in all cell fractions, but mostly in NCNI cell fractions and $\alpha\beta$ T cell fractions (Fig. 5B). IL-22 was detected mostly in $\alpha\beta$ T cell fractions (Fig. 5C). The IL-22 binding protein, which inhibited binding of IL-22 to the cellular receptor, was detected mostly in CD11b⁺ cell fractions (Fig. 5D). COX-2, PGES, IL-6, MCP-1 (13), and CINC-2 were clearly expressed in NCNI cell fractions (Table II). MIP-2 was clearly expressed in CD11b⁺ cell fractions but was thought to be slightly expressed also in NCNI cells ($8.4 \pm 2.8\%$), because *CD11b* gene expression in NCNI cell fractions, which was presumed to be due to contaminating CD11b⁺ cells, was $<1\%$ (13).

Characterization of cultivated noncardiomyocytic cells from EAM hearts

Immunohistochemical staining of cultured cells showed that anti-smooth muscle actin-positive, collagen type III-positive, and CD11b⁺ cells were the major populations and that factor VIII-positive cells were rare. A CD3⁺ cell was not observed in the slide (Fig. 6). Moreover, this finding is consistent with gene expression analysis of cultured cells, because collagen type III, calponin, and CD11b mRNA levels were high and von Willebrand factor mRNA levels were low. CD3 and α -cardiac myosin mRNA was not detected in cultivated cells (Table III). These results suggested that

the cultivated NC cells contained mainly fibroblasts, smooth muscle cells, and CD11b⁺ cells.

mRNA and protein expression of immunologic molecules in cultivated noncardiomyocytic cells from EAM hearts

Because IL-22-targeting cells in EAM hearts were NCNI cells and the genes of PGES, COX-2, CINC-2, MIP-2, IL-6, and MCP-1(13) were also expressed by NCNI cells (Table II), we examined the effect of IL-22 on expression of these genes in cultivated NC cells. Results showed that IL-22-Ig-containing serum or rIL-22, the concentration of which was lower than that in serum of IL-22-Ig-treated rats at any time, significantly inhibited gene expression of PGES, COX-2, CINC-2, MIP-2, IL-6, and MCP-1 in cultivated NC cells from EAM that were stimulated with IL-1 α (Figs. 7 and 8). These phenomena were observed in all three procedural experiments. Moreover, ELISA analysis of culture media containing IL-6, CINC-2 α , and MCP-1 showed that rIL-22 also significantly inhibited their protein expression (Fig. 9).

Discussion

Recent reports suggest diverse functions of IL-22. Similar to the results of this study, Radaeva et al. (17) reported that IL-22 ameliorated mouse T cell-mediated hepatitis. They proposed that the mechanisms of the effect were due to antiapoptotic and mitogenic

Table II. Absolute copy numbers of immunologic molecules mRNA in day 18 EAM hearts^a

	Copy Numbers of mRNA/ μ g of Total RNA (Copy Numbers of mRNA/the Most Copy Numbers of mRNA)							
	Cardiomyocytes ($n = 5$)		$\alpha\beta$ T Cells ($n = 5$)		CD11b ⁺ Cells ($n = 5$)		NCNI Cells ($n = 6$)	
COX-2	279,000 \pm 83,500	(0.93 \pm 0.28%)	863,000 \pm 373,000	(2.9 \pm 1.2%)	4,950,000 \pm 1,840,000	(16.5 \pm 6.1%)	30,000,000 \pm 6,720,000 ^b	(100 \pm 22.4%)
PGES	36,800 \pm 12,400	(8.9 \pm 3.0%)	28,000 \pm 22,600	(6.8 \pm 5.5%)	N.D.		414,000 \pm 87,500 ^b	(100 \pm 21.0%)
CINC2	88,000 \pm 25,600	(1.7 \pm 0.5%)	596,000 \pm 308,000	(11.6 \pm 6.0%)	3,100,000 \pm 1,080,500	(60.4 \pm 21.1%)	5,130,000 \pm 1,050,000	(100 \pm 20.5%)
MIP-2	824,000 \pm 339,000	(1.2 \pm 0.5%)	5,750,000 \pm 2,800,000	(8.6 \pm 4.2%)	66,700,000 \pm 30,800,000 ^c	(100 \pm 46.2%)	5,620,000 \pm 1,870,000	(8.4 \pm 2.8%)
IL-6	1,180,000 \pm 495,000	(1.8 \pm 0.7%)	752,000 \pm 435,000	(1.1 \pm 0.6%)	569,000 \pm 248,000 ^b	(0.8 \pm 0.4%)	67,300,000 \pm 19,200,000 ^c	(100 \pm 28.5%)

^a Immunologic molecules are listed in the far left column. Cardiomyocytes, $\alpha\beta$ T cells, CD11b⁺ cells, and NCNI cells from day 18 EAM hearts were purified and total RNA was isolated. The absolute copy numbers of these immunologic molecule RNA were measured by quantitative RT-PCR. Results are expressed as the mean \pm SEM. N.D., not detected.

^b $p < 0.01$ vs any other group.

^c $p < 0.05$ vs any other group.

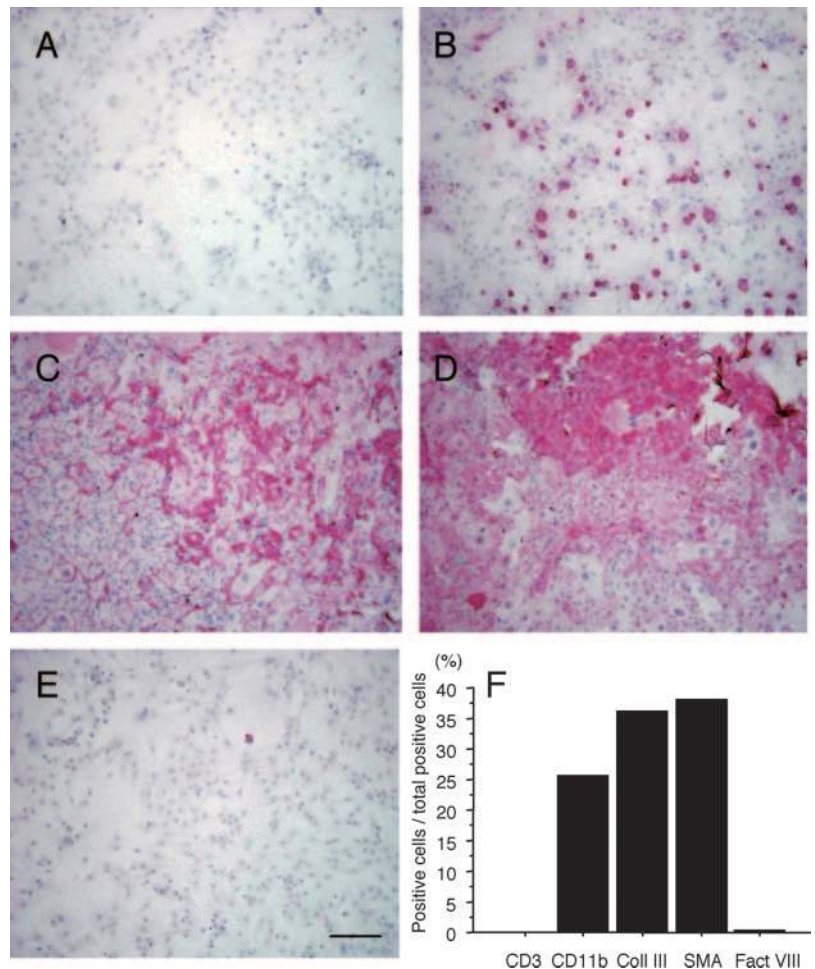


FIGURE 6. A–E, Immunohistochemical staining of cultured cells from EAM hearts. NC cells were isolated from the hearts of EAM rats on day 18 and cultivated for 2 wk on chamber slides. The NC cells were immunostained with Ab for CD3 (A), CD11b (B), collagen type III (C), α -smooth muscle actin (D), and factor VIII-related Ag (E). F, Positive cells of CD3, CD11b, collagen type III, α -smooth muscle actin, and factor VIII-related Ag in 1-mm² chamber slides were counted, and the numbers of each positive cell per the numbers of total positive cells were calculated. Bar represents 100 μ m. Coll III, collagen type III; SMA, α smooth muscle actin; Fact VIII, factor VIII related Ag.

actions on hepatocytes via the IL-22 receptor. Moreover, Whittington et al. (18) reported synergy between IL-10 and IL-22 in terms of IL-8 inhibition in an IL-22RA1-expressing human alveolar lung cell line and a role for IL-22 in the regulation of pulmonary inflammation. Conversely, there are also some reports of IL-22 as a proinflammatory cytokine that induced the production of MCP-1 in synovial fibroblasts (19) and increased inflammatory cytokines and chemokines in colonic subepithelial myofibroblasts (20). Boniface et al. (21) reported that IL-22 up-regulated the expression of S100A7, S100A8, and S100A9, a group of proinflammatory molecules belonging to the S100 family of calcium-binding proteins, as well as matrix metalloproteinase-3 of keratinocytes. IL-22 has been also reported to act mainly on var-

ious nonimmune tissue cells and to promote the innate, nonspecific immunity of tissues as a T cell mediator (22). Thus, there are diverse opinions on the role of IL-22.

In this study, we examined the effect of IL-22 on IL-1-stimulated, cultivated NCNI cells. Our previous study demonstrated the following: 1) CD11b⁺ cells in EAM hearts expressed massive amounts of IL-1 mRNA; and 2) NCNI cells in EAM hearts were IL-1-targeting cells as well as T cells (9). Because not only IL-22 but also IL-1 act on NCNI cells in EAM hearts, the effect of IL-22 on NCNI cells in EAM hearts is suspected to be more similar to the effect of IL-22 on IL-1-stimulated NCNI cells than on resting NCNI cells. It is well known that IL-1 or TNF- α , which is produced by CD11b⁺ (9, 13), enhances expression of COX-2 and PGES (23, 24). Because COX-2 and PGES were produced by NCNI cells as shown in this study, we suspected that IL-22 acted directly on NCNI cells and suppressed the production of COX-2 and PGES in IL-1-stimulated NCNI cells. COX-2 is recognized as a major contributor to peripheral inflammatory processes, and its selective inhibition has become a mainstay therapy for rheumatoid and osteoarthritis (25). PGES is up-regulated in vivo after LPS or adjuvant administration and is a key enzyme involved in the formation of PGE₂ in COX-2-mediated inflammatory and pyretic responses (26, 27). Of the prostaglandins generated by COX-2 activity, PGE₂ appears to be the principal mediator of peripheral inflammation (28). Our results from the suppression of COX-2 and PGES by rIL-22 and serum containing IL-22-Ig suggest a possible mechanism that could ameliorate EAM by IL-22-Ig.

Moreover, it is also well known that the production of chemokines such as CINC-2, MIP-2, MCP-1, and IL-6 are induced by IL-1 or TNF- α in fibroblasts (29–31). Chemokines are reasonable therapeutic

Table III. Absolute copy numbers of specific cell marker mRNA in cultivated cells from EAM hearts^a

	Copy Numbers of mRNA/ μ g of Total RNA NC Cells (n = 5)
CD3	N.D.
Collagen type III	751,000,000 \pm 100,000,000
Calponin	296,000,000 \pm 43,200,000
CD11b	22,200,000 \pm 4,770,000
von Willebrand factor	1,090,000 \pm 260,000
α -Cardiac myosin	N.D.

^a NC cells from day 18 EAM hearts were cultured for 2 wk. After reaching confluency, NC cells were collected and total RNA was isolated. The absolute copy numbers of CD3, collagen type III, calponin, CD11b, von Willebrand factor, and anti-cardiac myosin mRNA (n = 5) were measured by quantitative RT-PCR. Results are expressed as the mean \pm SEM. N.D., not detected.

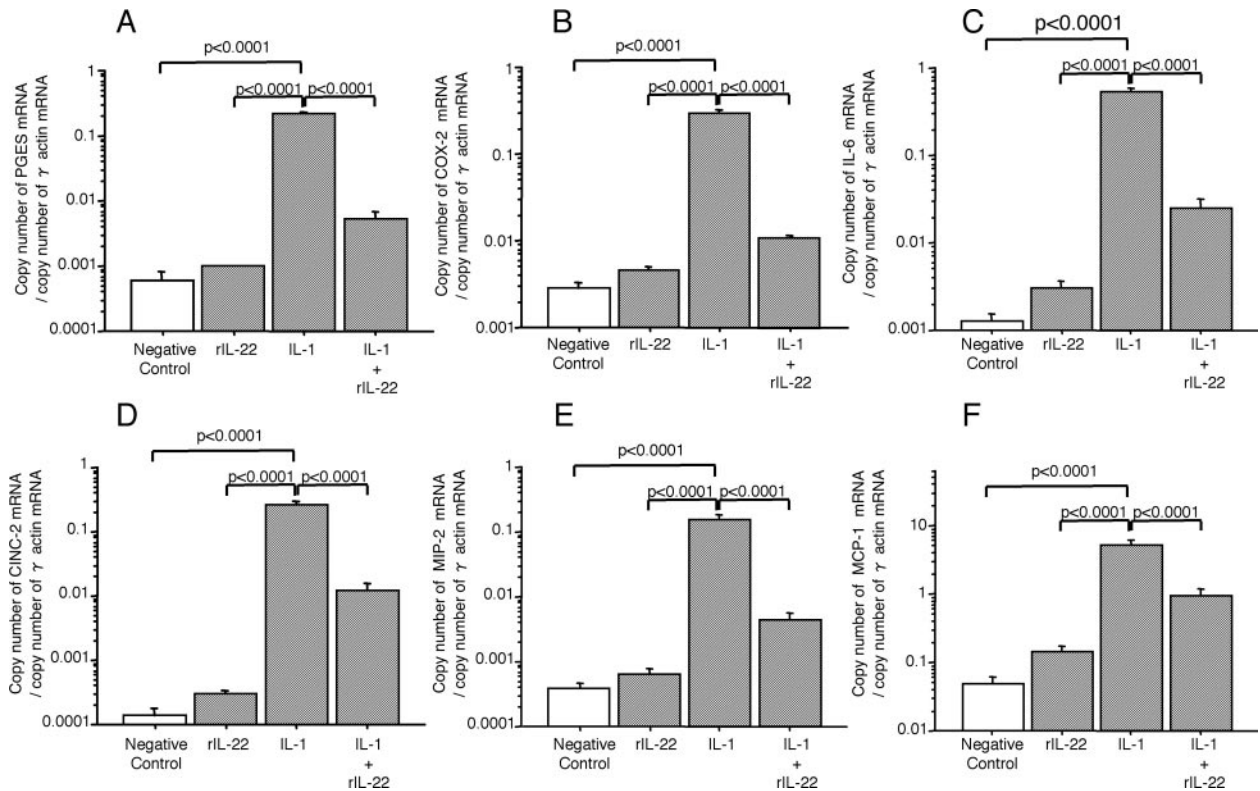


FIGURE 7. Copy numbers of various immunological molecules mRNA per copy numbers of γ -actin mRNA in cultivated NC cells ($n = 5$) from EAM hearts. NC cells are mainly fibroblasts, smooth muscle cells, endothelial cells, and CD11b⁺ cells. The mRNA measured were for PGES (A), COX-2 (B), IL-6 (C), CINC-2 (D), MIP-2 (E), and MCP-1 (F). Columns represent the following: Negative Control, cells cultivated in 2 ml of RPMI 1640 medium containing neither IL-1 α nor rIL-22; rIL-22, cells cultivated in RPMI 1640 medium containing only rIL-22 (10 ng/ml); IL-1, cells cultivated in RPMI 1640 medium containing only IL-1 α (1 ng/ml); IL-1 + rIL-22, cells cultivated in RPMI 1640 medium containing IL-1 α (1 ng/ml) and rIL-22 (10 ng/ml). Error bars represent SEM. Statistical assessment was performed by one-way ANOVA and Bonferroni's multiple comparison test. Differences were considered significant at $p < 0.05$. Results are representative of three independent experiments.

targets in autoimmunity (32), and administration of neutralizing Abs to IL-6 reduces autoimmune diseases (33). Because NCNI cells in EAM hearts produce these chemokines and IL-6 as shown in this study and from our previous data (13), IL-22 directly suppresses the production of these immunological proteins promoted by IL-1 and may ameliorate EAM. Rat CINC-2 and MIP-2 are thought to be counterparts of human IL-8 (34). Our results of the suppression of CINC-2 and MIP-2 by IL-22 in IL-1-stimulated NC cells from EAM hearts may be concurrent with the suppression of IL-8 by IL-22 in a human alveolar lung cell line (18).

However, some of the above-mentioned reports that propose that IL-22 may induce MCP-1 in synovial fibroblasts from rheumatoid arthritis patients (19) and increase IL-6, IL-8, IL-11, leukemia inhibitory factor, and chemokines in colonic subepithelial myofibroblasts (20) are seemingly inconsistent with our results. The discrepancy may be explained by two reasons. One reason is that IL-22 may function differently in cells from different tissues or diseases. We examined the effect of IL-22 on IL-1-stimulated NC cells from normal rat hearts, but IL-22 did not affect the increase of COX-2, PGES, and MCP-1 mRNA expression induced by IL-1 (data not shown). The other reason may be due to IL-1 stimulation. We demonstrated that IL-22 suppressed up-regulation of some immunological molecule mRNA and protein expression by IL-1 stimulation in cultured NC cells from EAM hearts. We also examined gene expression of COX-2, PGES, CINC-2, MIP-2, MCP-1, and IL-6 in IL-1-nonstimulated NC cells to investigate the effect of IL-22 on resting NC cells from EAM hearts. The differences in gene expression between the negative control and the IL-22 group of IL-1-nonstimulated NC cells were not significant

according to one-way ANOVA and Bonferroni's multiple comparison test, but gene expression tended to be inversely higher in the IL-22 group cells than in the negative control cells. Moreover, we examined the effect of IL-22 in TNF- α -stimulated NC cells from EAM hearts, but IL-22 did not suppress COX-2, PGES, and MCP-1 mRNA expression (data not shown). Therefore, this result means that IL-22 may suppress gene expression only in the case of IL-1-stimulated NC cells cultured from the lesion site. The signal transduction pathway of IL-22 is assumed to be similar to that of IL-10 (35). IL-10 has been reported to suppress MCP-1 in IL-1- or TNF- α -stimulated intestinal epithelial cells (36) and to inhibit IL-6 in LPS-stimulated gingival fibroblasts (37) and IL-6 and MIP-2 in TNF- α -stimulated corneal epithelial cells and fibroblasts (38). IL-22 may act only on IL-1-stimulated cells from EAM hearts as a regulator of the expressions of these genes, similarly as IL-10. Further study is needed to determine the effect of IL-22 under several conditions.

Previous reports showed that IL-10, which is structurally similar to IL-22, ameliorated several autoimmune diseases (5, 39, 40). However, IL-22-producing and -targeting cells have been reported to be different from those of IL-10 (35). We have previously reported that IL-10-producing cells in EAM hearts were mainly NCNI cells and that IL-10-targeting cells were mainly $\alpha\beta$ T cells and CD11b⁺ cells, although to some extent NCNI cells also expressed the gene of the IL-10R α (13). This result demonstrated that IL-10 could directly regulate the production of proinflammatory cytokines by immune cells. By contrast, in this study IL-22 producing cells were thought to be $\alpha\beta$ T cells, and IL-22 targeting cells were thought to be NCNI cells but not immune cells. Wolk et

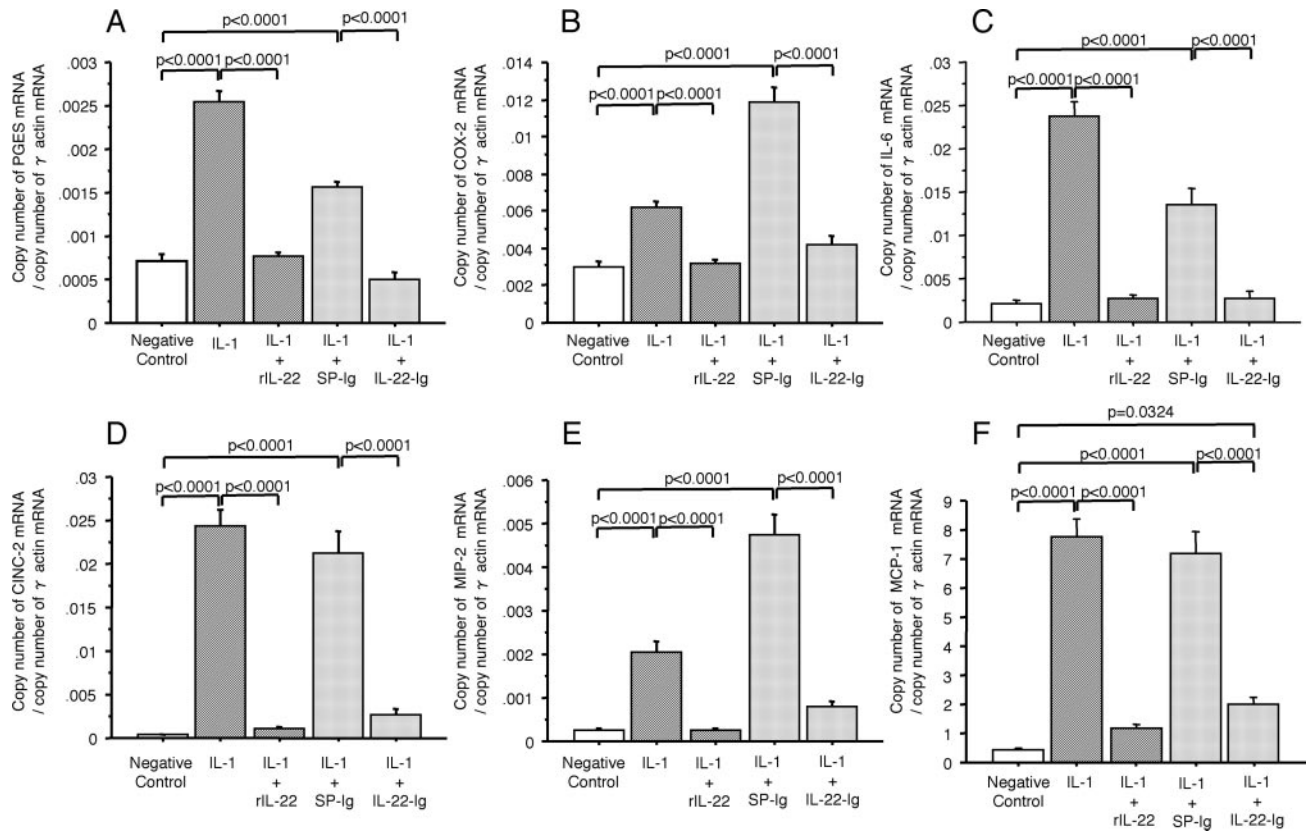


FIGURE 8. Copy numbers of various immunological molecules mRNA per copy numbers of γ actin mRNA in cultivated NC cells ($n = 5$) from EAM hearts. The mRNA measured were for PGES (A), COX-2 (B), IL-6 (C), CINC-2 (D), MIP-2 (E), and MCP-1 (E). Columns represent the following: Negative Control, cells cultivated in 2 ml of RPMI 1640 medium containing no IL-1 α , rIL-22, or rat serum; IL-1, cells cultivated in 2 ml of RPMI 1640 medium containing only IL-1 α (1 ng/ml); IL-1 + rIL-22, cells cultivated in 2 ml of RPMI 1640 medium containing IL-1 α (1 ng/ml) and rIL-22 (10 ng/ml); IL-1 + SP-Ig, cells cultivated in 2 ml of RPMI 1640 medium containing IL-1 α (1 ng/ml) and 100 μ l of serum obtained from SP-Ig treated EAM rats on day 17; IL-1 + IL-22-Ig, cells cultivated in 2 ml of medium containing IL-1 α (1 ng/ml) and 100 μ l of serum obtained from IL-22-Ig-treated EAM rats on day 17 (6 nmol/L \approx 260 ng/ml). The final concentration of the IL-22-Ig protein in the culture dish was 0.3 nmol/L. At any time point, the concentration of the IL-22-Ig protein was lower than that in serum of IL-22-Ig-treated EAM rats. Error bars represent SEM. Statistical assessment was performed by one-way ANOVA and Bonferroni's multiple comparison test (negative control vs IL-1 vs IL-1 + rIL-22 or negative control vs IL-1 + SP-Ig vs IL-1 + IL-22-Ig). Differences were considered significant at $p < 0.05$. Results are representative of three or more independent experiments.

al. (22) reported that neither resting nor activated immune cells expressed the IL-22 receptor and that IL-22 did not have any effects on these cells. IL-22 has not been reported to inhibit the production of proinflammatory cytokines by monocytes, namely

TNF- α or IL-1 (4). TNF- α - and IL-1-producing cells in EAM hearts were thought to be CD11b $^{+}$ cells (9, 13). These findings are consistent with our results. We previously reported that IL-1R antagonist-Ig (9), IL-13-Ig (14), and CTLA4-Ig (41) ameliorated EAM, but

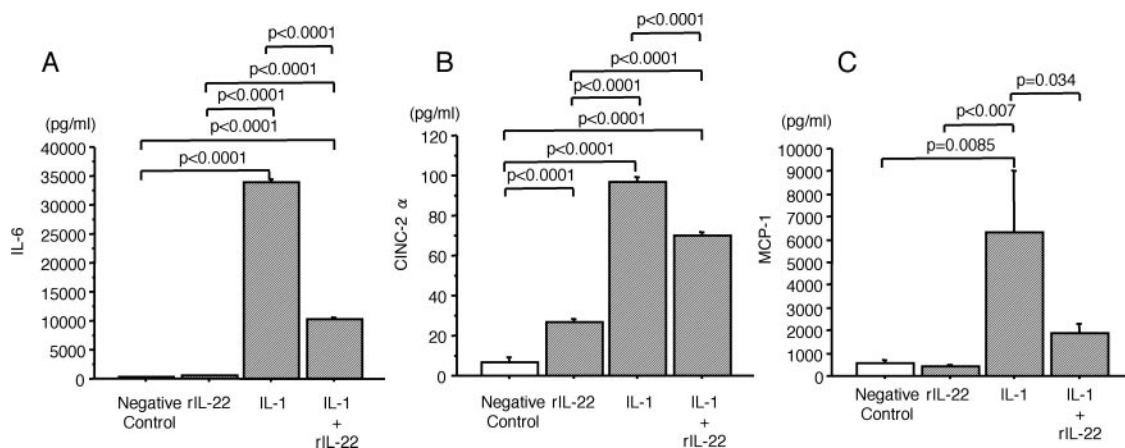


FIGURE 9. ELISA of culture medium in NC cells ($n = 5$) cultivated from EAM hearts. NC cells were cultivated as shown in Fig. 7. Molecules examined were IL-6 (A), CINC-2 α (B), and MCP-1 (C). Error bars represent SEM. Statistical assessment was performed by one-way ANOVA and Bonferroni's multiple comparison test. Differences were considered significant at $p < 0.05$.

to some extent these proteins were thought to act on immune cells. However, this study demonstrated that IL-22, not acting on inflammatory cells, ameliorated EAM. In other words, amelioration of EAM by IL-22 shows that NCNI cells in EAM hearts or in other tissues besides the heart play an important role in the development of EAM. During the course of EAM, IL-1 and other inflammatory cytokines may be released into the circulation and have systemic effects. The gene therapy approach used is systemic. Therefore, the IL-22 therapy may influence IL-1 effects on endothelial cells, fibroblasts, smooth muscle cells, etc. in other tissues besides the heart. In any case, our present study indicates that we should actively attend to NCNI cells to understand the mechanism of EAM.

In summary, our data provide further evidence for an aspect of biological activities of IL-22 on autoimmune diseases. Our results also suggest that IL-22 acts mainly on NCNI cells in EAM hearts or in other tissues besides the heart and inhibits the production of some chemokines, cytokines, and prostaglandins in IL-1-stimulated NCNI cells from EAM hearts. Its action mechanism may explain the amelioration of rat EAM by IL-22.

Disclosures

The authors have no financial conflict of interest.

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