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Chronic Inflammatory Cardiomyopathy of Interferon γ -Overexpressing Transgenic Mice Is Mediated by Tumor Necrosis Factor- α

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We recently described a model of inflammatory cardiomyopathy in interferon (IFN)- γ overexpressing transgenic mice stably circulating IFN- γ in the serum referred to as SAP-IFN- γ mice. SAP-IFN- γ transgenic mice show cardiac infiltration by mononuclear leukocytes, culminating in dilated cardiomyopathy characterized by an increase of left ventricular end diastolic diameter and reduction of fractional shortening. We hypothesized that the pathological mechanism underlying SAP-IFN- γ cardiomyopathy might be mediated by (auto)immune processes or tumor necrosis factor (TNF)- α synthesis from IFN- γ -activated macrophages. To verify these hypotheses, we crossed SAP-IFN- γ transgenic mice with immunodeficient $\text{Rag1}^{-/-}$ or $\text{TNF-}\alpha^{-/-}$ knockout mice and analyzed the cardiac phenotype of the resulting double-mutant offspring. Immunodeficient $\text{Rag1}^{-/-}$ SAP-IFN- γ mice had a decreased impaired life span and intensive cardiac inflammatory reactions, showing that the cardiotoxic IFN- γ effect operative in SAP-IFN- γ mice was not mediated by an adaptive immune mechanism. SAP-IFN- γ $\text{TNF-}\alpha^{-/-}$ hearts showed virtually no histopathological alterations, a significant reduction of cardiac infiltration by CD11c^+ dendritic cells and F4/80^+ macrophages, almost complete normalization of cardiac troponin T levels in serum and of left ventricular end

diastolic diameter and fractional shortening, and a dramatic increase of life span, compared with SAP-IFN- γ transgenic controls. Thus, myocarditis and cardiomyopathy developing in IFN- γ -overexpressing transgenic mice is, to a significant degree, mediated by TNF- α . TNF- α -mediated cardiotoxicity in SAP-IFN- γ transgenic mice is independent of changes of apoptosis. (*Am J Pathol* 2012, 180:73–81; DOI: 10.1016/j.ajpath.2011.09.006)

Activation of the immune system is thought to contribute to the pathogenesis of chronic heart failure (CHF). In a multitude of clinical studies, infiltration of the cardiac tissue by leukocytes, cellular activation of specific immunocytes, and increased levels of tumor necrosis factor (TNF)- α , IL-1 β , and IL-6 in plasma and myocardium could frequently be detected in patients with CHF.^{1–9} Thus, the network of inflammatory cytokines is considered an important contributor to the pathogenesis of CHF. Because TNF- α exerts clear cardiotoxic properties in various models of overexpressing transgenic mice,^{10,11} this pro-inflammatory cytokine is considered a particularly interesting mediator of chronic cardiac disease. However, simplistic therapeutic approaches using TNF- α antagonistic antibodies for CHF treatment have led to negative, or at least contradictory, results, clearly indicating the need for further research in the field of immunopathogenesis of CHF.^{12,13}

Interferon (IFN)- γ represents another cytokine with pleiotropic pro-inflammatory effects. Although this cytokine is a potent inducer of TNF- α synthesis,¹⁴ there is only

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minor clinical evidence for a contribution of this protein to chronic human cardiomyopathy. In an attempt to clarify the role of IFN- γ in chronic heart disease, we recently published a transgenic mouse model of IFN- γ -mediated cardiomyopathy.¹⁵ The transgenic mice used for this study have integrated a fusion gene composed of serum amyloid P-component (Apcs, old symbol SAP) promoter sequences and murine IFN- γ cDNA and consequently show massive liver-specific overexpression and constitutive serum circulation of IFN- γ .¹⁶ SAP-IFN- γ mice have developed chronic active hepatitis and myocarditis progressing to cardiomyopathy.^{15,16} The cardiac phenotype of SAP-IFN- γ mice was characterized by progressive infiltrations with CD4⁺ and CD8⁺ T cells and Mac2⁺ macrophages and high-level monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , and TNF- α expression.

The current study investigated the mechanisms underlying cardiac pathogenesis of SAP-IFN- γ transgenic mice. To this end, we crossed the SAP-IFN- γ mice with immunodeficient *Rag1*^{+/-} animals. The resulting *Rag1* SAP-IFN- γ mice exhibited cardiac inflammatory reactions and showed a dramatic impairment of life expectancy compared with immunocompetent *Rag1*^{+/-} SAP-IFN- γ controls, clearly indicating that myocarditis induced by IFN- γ overexpression is not mediated by (auto)immune responses but that adaptive immunity exerts a protective effect on the phenotype of the SAP-IFN- γ mice. Because SAP-IFN- γ disease had been associated with cardiac macrophage and dendritic cell infiltrations and TNF- α expression,¹⁵ the cardiotoxic IFN- γ effects might be induced by excessive TNF- α synthesis of IFN- γ -activated cardiac macrophages or dendritic cells. We, thus, crossed SAP-IFN- γ transgenic mice with TNF- α ^{-/-} knockout mice¹⁸ and analyzed the resulting double-mutant mice. Functional echocardiographic analyses revealed that the TNF- α gene defect led to an almost complete normalization of left ventricular end diastolic diameter (LVEDD) and fractional shortening (FS) of SAP-IFN- γ hearts. In full accord with this result, histopathological alterations and cardiac infiltration by CD11c⁺ dendritic cells and F4/80⁺ macrophages and serum cardiac troponin T (cTNT) levels were significantly reduced in the double-mutant mice compared with SAP-IFN- γ transgenic control hearts. These results demonstrate that myocarditis and cardiomyopathy developing in IFN- γ -overexpressing transgenic mice are, at least to a significant amount, mediated by TNF- α , most probably synthesized by IFN- γ -activated macrophages or dendritic cells.

Materials and Methods

Mouse Breeds

The production and characteristics of transgenic-lineage SAP-IFN- γ 5 (official strain designation Tg(APCS-IIfng)5Imeg, hereafter referred to as SAP-IFN- γ) have been previously described.¹⁶ Briefly, SAP-IFN- γ transgenic mice carry the cDNA of the murine IFN- γ gene

cloned downstream from the liver-specific promoter of the human serum amyloid P component (SAP) gene. The SAP-IFN- γ strain used was adequately backcrossed to the C57BL/6J (B6) inbred strain.¹⁹

Rag1^{-/-} SAP-IFN- γ and *Rag1*^{+/-} experimental animals were generated by crossing *Rag1*^{-/-} SAP-IFN- γ males and *Rag1*^{-/-} females (official strain designation: B6.129S7-*Rag1*<tm1Mom>; The Jackson Laboratory, Bar Harbor, ME). *Rag1*^{+/-} SAP-IFN- γ and *Rag1*^{+/-} mice were bred by mating SAP-IFN- γ and *Rag1*^{-/-} mice.

We further established a cross of TNF- α ^{-/-} SAP-IFN- γ males and TNF- α ^{-/-} females (official strain designation: B6.129S-Tnf<tm1Gkl>, stock 005540 The Jackson Laboratory) to obtain TNF- α ^{-/-} SAP-IFN- γ and TNF- α ^{-/-} experimental animals. TNF- α ^{+/-} SAP-IFN- γ and TNF- α ^{+/-} experimental mice were generated by mating SAP-IFN- γ and TNF- α ^{-/-} mice.

Transgenic offspring could be discriminated from their nontransgenic littermates by PCR amplification, as previously described.¹⁹ The TNF- α and *Rag1* loci were genotyped as described in the protocol of The Jackson Laboratory. Mice were housed in the Central Laboratory Animal Facility of the University of Mainz (Mainz, Germany) under specified pathogen-free conditions.

Histological Analyses

Mice were sacrificed by exposure to CO₂, and cardiac tissue specimens were fixed in 4% buffered formaldehyde solution. Sections were immediately embedded in paraffin, and sections (4 μ m thick) were stained with H&E or Masson's trichrome, according to standard protocols.

Apoptosis was analyzed using the *In Situ* Cell Death Detection Kit, AP (Roche, Basel, Switzerland) for immunohistochemical detection and quantification of apoptosis, based on labeling of DNA strand breaks (TUNEL technology), according to the manufacturer's instructions. The number of apoptotic cells was counted in 10 microscopic high-power fields (HPFs) per mouse, and the mean number of apoptotic cells per HPF was calculated for each animal group.

Immunofluorescence Analyses

Cryosections were prepared from snap-frozen murine organs and analyzed by immunofluorescence staining. Immunofluorescence was performed using the TSA Cy3 system (Perkin Elmer, Rodgau, Germany) and a fluorescence microscope (Olympus fluorescence microscope; Olympus, Melville, NY). In brief, cryosections were fixed in paraformaldehyde for 10 minutes, followed by sequential incubation with methanol, avidin-biotin (Vector Laboratories, Burlingame, CA), and protein blocking reagent (Dako, Wiesbaden, Germany) to eliminate unspecific background staining. Slides were then incubated overnight with primary antibodies specific for CD11c, CD4 (BD Bioscience, Heidelberg, Germany), or F4/80 (MD Bioscience, St Paul, MN). Subsequently, the slides were incubated for 30 minutes at room temperature with biotinylated secondary antibodies (Dianova, Darmstadt,

Germany). All samples were finally treated with streptavidin-horseradish peroxidase and stained with tyramide (Cy3), according to the manufacturer's instructions (Perkin Elmer). Before examination, the nuclei were counterstained with Hoechst3342 (Molecular Probes, Eugene, OH). Positive cells were counted per HPF.

ALT and cTNT Determination

Alanine transaminase (ALT) was quantitated in murine serum using the Scil Reflovet Plus system (Scil Animal Care Company, Viernheim, Germany). cTNT was measured with the Elecsys Troponin T hs test (MODULAR ANALYTICS E170, Roche Diagnostics GmbH, Mannheim, Germany) on a cobas e 601 analyzer (Roche Diagnostics GmbH).²⁰ The detection limit of the assay was 5 pg/mL.

RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from heart samples (0.5 to 1.0 g) by acid guanidinium thiocyanate-phenol-chloroform extraction, as described.²¹ A one-step RT-PCR was performed in 25- μ L reactions in a 96-well spectrofluorometric thermal cycler (iCycler; Bio-Rad, München, Germany). For these real-time quantitative RT-PCR analyses (30 minutes at 50°C, 15 minutes at 95°C, and 40 cycles of 15 seconds at 94°C and 60 seconds at 60°C), the following oligonucleotides listed served as sense and antisense primers and TaqMan hybridization probes: IL12-p40 (sense, 5'-TACTCCGGA-CGGTTCACGTG-3'; antisense, 5'-GTCACTGCCCCGAGAGTCAGG-3'; probe, 5'-AAGAAACATGGACTTGAAGTTCAACATCAAGAGCA-3'), MCP-1 (sense, 5'-CTTCTGGGCCTGCTGTTC-3'; antisense, 5'-CCAGCCTACTCATTGGGATC-3'; probe, 5'-CTCAGCCAGATGCAGTTAACGCCC-3'), MIP-1 α (sense, 5'-CTGCAACCAAGTCTTCTCAGC-3'; antisense, 5'-CTGCCTCCAAGACTCTCAGG-3'; probe, 5'-ACTGCCTGCTGCTTCTCTACAGCC-3'), TNF- α (sense, 5'-TCTTCTCATTCTGCTTGTGG-3'; antisense, 5'-ATGAGAGGAGGCCATTG-3'; probe, 5'-TACTGAACTTCGGGGTGATCGGTCC-3', which specifically detects the part of the TNF- α mRNA deleted in the knockout animals), and murine RNA polymerase II subunit A (mPOL2A) (sense, 5'-ACCACGTCCAATGATATTGTGGAG-3'; antisense, 5'-ATGTCATAGTGTCACACAGGAGCG-3'; probe, 5'-CTGGGCATTGAGGCTGTGCGGAA-3').

TaqMan hybridization probes were double labeled with 6-carboxyfluorescein as reporter fluorophore and carboxy tetramethyl-rhodamine as quencher. All primers and dual-labeled probes (5'-6-carboxyfluorescein, 3'-carboxy tetramethyl-rhodamine) were from MWG-Biotech, Ebersberg, Germany. Fluorescence was monitored at each 60°C step. Each experimental reaction was performed in triplicate. All primer-probes sets had efficiencies of 100% ($\pm 10\%$). To calculate the relative expression of IL12-p40, MCP-1, MIP-1 α , and TNF- α mRNA in the animals, the $2^{(-\Delta\Delta C_T)}$ method²² was used. According to this method, the C_T values for IL12-p40, MCP-1, MIP-1 α , and TNF- α mRNA expression in each sample were normalized to the C_T values of mPOL2A mRNA in the same

sample. The $2^{(-\Delta\Delta C_T)}$ values of the TNF- $\alpha^{+/-}$ mice for each mRNA analyzed were set to 100%.

Mouse Echocardiography

Echocardiographic analyses were performed in anesthesia, as previously described.¹⁵ The percentage FS was calculated as follows: [(LVEDD-left ventricular end systolic diameter)/LVEDD] $\times 100$.²³

Statistical Analysis

All data are presented as mean $\pm$ SD. Statistical differences were determined either by the Student's *t*-test or factorial analysis of variance, followed by the Fisher's protected least-significant-difference test for comparison of multiple means.

Results

SAP-IFN- γ Cardiomyopathy Is Not Mediated by an Adaptive Autoimmune Mechanism

Because IFN- γ is a cytokine with multiple pro-inflammatory effects on adaptive immunity and because abundant T cells and CD11c⁺ dendritic cells with the capacity to regulate T-cell immunity^{24,25} have been detected in the hearts of SAP-IFN- γ mutants,¹⁵ the cardiac pathomechanism induced by the pro-inflammatory cytokine IFN- γ may be mediated by an adaptive (auto)immune response. To test for this hypothesis, the SAP-IFN- γ transgenic strain was crossed to the *Rag1*^{-/-} immunodeficient knockout variant that completely lacks T and B cells.¹⁷ The resulting *Rag1*^{-/-} SAP-IFN- γ double-mutant mice and their *Rag1*^{+/-} SAP-IFN- γ , *Rag1*, and *Rag1*^{+/-} littermates were subjected to a long-term survival study, and Kaplan-Meier plots were generated. As depicted in Figure 1, virtually all *Rag1*^{+/-} SAP-IFN- γ mice died within the age span of 6 and 12 months, which is in full accord with the data previously described for SAP-IFN- γ mice.^{15,16} To our surprise, the life span of *Rag1*^{-/-} SAP-IFN- γ mice was significantly impaired, clearly indicating that the adaptive immune system has a beneficial effect on SAP-IFN- γ life expectancy and excluding the possibility that myocarditis of SAP-IFN- γ mice might be induced by autoimmunity. In line with this result, intensive inflammatory reactions could be observed in the hearts of immunodeficient *Rag1*^{-/-} SAP-IFN- γ mice (data not shown), suggesting that the cardiotoxic IFN- γ effect, as observed in SAP-IFN- γ mice, was not mediated by an adaptive immune mechanism.

Normalization of Cardiac Phenotype and cTNT Levels of SAP-IFN- γ Mice by TNF- α Inactivation

To further investigate the pathomechanisms leading to myocarditis and cardiomyopathy in IFN- γ -overexpressing SAP-IFN- γ mice,¹⁵ the SAP-IFN- γ transgenic strain

Figure 1. Kaplan-Meier plots. The survival rates of *Rag1*^{+/-} *SAP-IFN-γ* and *Rag1*^{-/-} *SAP-IFN-γ* mice and *TNF-α*^{+/-} *SAP-IFN-γ* and *TNF-α*^{-/-} *SAP-IFN-γ* mice were compared in a long-term study. The life expectancy of *Rag1*^{+/-} *SAP-IFN-γ* (data not shown) was comparable to that of *TNF-α*^{+/-} *SAP-IFN-γ* mice (circles). *Rag1*^{+/-} and *Rag1*^{-/-} and *TNF-α*^{+/-} and *TNF-α*^{-/-} control mice did not show any significant mortality before the age of 12 months (data not shown). Note the detrimental effect of the *Rag1* gene defect (triangles) and the beneficial effect of the *TNF-α* gene defect (squares) on *SAP-IFN-γ* life expectancy. Data shown represent Kaplan-Meier plots of at least 10 animals per genotype.

was crossed to the *TNF-α* knockout strain and the cardiac phenotype of *TNF-α*^{-/-} *SAP-IFN-γ* double-mutant mice was compared with that of isogenic *TNF-α*^{+/-} *SAP-IFN-γ*-positive controls. As controls, we used age-matched *TNF-α*^{-/-} and *TNF-α*^{+/-} mice. Most mice were sacrificed at the age of 6 months because older *SAP-IFN-γ* animals have a restricted life expectancy.¹⁵ In addition, we used mice aged 9 months only for echocardiographic analyses.

Because all mice used in the current study had the same C57BL/6J genetic background as those used in our previous work¹⁵ and because the *TNF-α* knockout allele fails to induce any significant phenotype under heterozygous conditions,¹⁸ we assumed that *TNF-α*^{+/-} *SAP-IFN-γ* mice would show the same cardiac phenotype as previously described for *SAP-IFN-γ* transgenic mice.¹⁵ In full accord with this expectation, we found the same cardiac alterations in the *TNF-α*^{+/-} *SAP-IFN-γ* mice of the current study (Figure 2, A and B), as previously observed for 6-month-old *SAP-IFN-γ* mice.¹⁵ Characteristic cardiac histological features of both genotypes were dropout and variation in the size and shape of cardiac muscle fibers, intensive mononuclear cell infiltrations, and reactive interstitial fibrosis. In contrast, the cardiac abnormalities of the *TNF-α*^{-/-} *SAP-IFN-γ* mice, combining IFN-γ overexpression with the homozygous *TNF-α* knockout defect, were much more discrete (Figure 2, A and B), with significant reduction of cardiomyolysis and interstitial mononuclear infiltrate. No pathological changes could be detected in the *TNF-α*^{+/-} and the *TNF-α*^{-/-} controls (Figure 2, A and B).

To further characterize the inflammatory infiltrates in the hearts of *TNF-α*^{+/-} *SAP-IFN-γ* and *TNF-α*^{-/-} *SAP-IFN-γ* transgenic animals, we performed quantitative immunofluorescence analyses with murine cryosections using anti-CD4-, anti-CD11c-, and anti-F4/80-specific antibodies (Figure 3). These analyses showed that mac-

rophages represent the most prominent leukocyte infiltrate in *TNF-α*^{+/-} *SAP-IFN-γ* hearts (24 cells per HPF, Figure 3B). In contrast, CD11c⁺ dendritic cells (17 cells per HPF, Figure 3A) and CD4⁺ T cells (eight cells per HPF, Figure 3C) contributed to the cardiac infiltrations to a significantly lower extent. For all immunomarkers investigated (Figure 3, A–C), infiltration by inflammatory cells was reduced in *TNF-α*^{-/-} *SAP-IFN-γ* hearts compared with the *TNF-α*^{+/-} *SAP-IFN-γ* controls. The anti-inflammatory effect mediated by the *TNF-α* knockout defect was most pronounced regarding macrophages (Figure 3B, Student's *t*-test: *P* < 0.01) and less prominent considering dendritic cells (Figure 3A, Student's *t*-test: *P* < 0.05) but failed to reach statistical significance regarding CD4⁺ T cells (Figure 3C). In Figure 3D, the experimental trend that the *TNF-α* knockout defect has the potential to virtually completely

Figure 2. Normalization of cardiac phenotype of *SAP-IFN-γ* mice by introduction of the *TNF-α* knockout defect. Representative sections from 6-month-old *TNF-α*^{+/-} *SAP-IFN-γ* (top left panel), *TNF-α*^{-/-} *SAP-IFN-γ* (top right panel), *TNF-α*^{+/-} (bottom left panel), and *TNF-α*^{-/-} (bottom right panel) mice stained with H&E (A) or Masson's trichrome (B) are shown. In accord with our previous results,¹⁵ interstitial mononuclear infiltrates (A, top left panel, arrowhead), degenerating muscle fibers (A, top left panel, arrow), and interstitial fibrosis (B, top left panel) could be observed in the hearts of *TNF-α*^{+/-} *SAP-IFN-γ* mice with an intact *TNF-α* allele. Virtually no such pathological changes could be observed in *TNF-α*^{-/-} *SAP-IFN-γ* hearts combining transgenic IFN-γ overexpression with the constitutive homozygous *TNF-α* knockout defect. Eight mice were evaluated per genotype. The slides depicted show characteristic cardiac regions of the respective genetic group.

Figure 3. Cellular characterization of myocarditis. Snap-frozen hearts prepared from 6-month-old male TNF- $\alpha^{+/+}$, TNF- $\alpha^{-/-}$, TNF- $\alpha^{+/+}$ SAP-IFN- γ , and TNF- $\alpha^{-/-}$ SAP-IFN- γ mice were stained with a set of leukocyte-specific antibodies and fluorescent secondary antibodies. In accord with our previous report,¹⁵ myocarditis of TNF- $\alpha^{+/+}$ SAP-IFN- γ transgenic mice with an intact TNF- α allele was characterized by significant infiltration by CD11c⁺ dendritic cells (A), F4/80⁺ macrophages (B), and CD4⁺ T cells (C). Introduction of the homozygous TNF- α knockout defect strongly reduced the infiltrative inflammatory reactions of SAP-IFN- γ hearts. The anti-inflammatory effect was statistically significant regarding dendritic cells (A) and macrophages (B). Virtually no specific staining reactions could be found in the hearts of the nontransgenic TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ controls. The data shown in A, B, and C represent mean $\pm$ SD values of five TNF- $\alpha^{+/+}$ SAP-IFN- γ and TNF- $\alpha^{-/-}$ SAP-IFN- γ animals and three TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice, respectively. The data of TNF- $\alpha^{+/+}$ SAP-IFN- γ and TNF- $\alpha^{-/-}$ SAP-IFN- γ animals were compared by the Student's t-test. ns indicates not significant. * $P < 0.05$, ** $P < 0.01$. D: The characteristic cardiac F4/80 staining reaction of a TNF- $\alpha^{+/+}$ SAP-IFN- γ (top panel) and a TNF- $\alpha^{-/-}$ SAP-IFN- γ (bottom panel) mouse. The TNF- α knockout defect has led to a virtually complete suppression of macrophage infiltration into the cardiac tissue of SAP-IFN- γ mice.

inhibit the infiltration of SAP-IFN- γ hearts by macrophages is impressively shown *in situ*.

In a further approach, we analyzed cTNT serum concentrations of TNF- $\alpha^{+/+}$ SAP-IFN- γ , TNF- $\alpha^{-/-}$, SAP-IFN- γ , TNF- $\alpha^{+/+}$, and TNF- $\alpha^{-/-}$ mice (Figure 4). In full accord with the cardiac phenotype of myocarditis and cardiomyopathy, serum cTNT concentrations of TNF- $\alpha^{+/+}$ SAP-IFN- γ mice were significantly increased compared with those of TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ control mice. Interestingly, cTNT concentrations of the TNF- $\alpha^{-/-}$ SAP-IFN- γ mice, combining IFN- γ overexpression and TNF- α gene deficiency, were still increased compared with those of the TNF- $\alpha^{+/+}$ and

TNF- $\alpha^{-/-}$ controls, but the values did not attain the order of magnitude of the TNF- $\alpha^{+/+}$ SAP-IFN- γ mice. The intermediate cTNT serum levels of the TNF- $\alpha^{-/-}$ SAP-IFN- γ mice clearly corroborate the normalization of cardiac phenotype of SAP-IFN- γ mice, as mediated by TNF- α gene inactivation.

Phenotypic Normalization of TNF- $\alpha^{-/-}$ SAP-IFN- γ Hearts Is Not Associated with Normalization of Pro-Inflammatory Cytokine Transcription

In our previous report,¹⁵ we showed that myocarditis of SAP-IFN- γ transgenic hearts is associated with high-level expression of pro-inflammatory cytokines MCP-1, MIP-1 α , TNF- α , and IL12-p40. Because constitutive inactivation of TNF- α gene expression had normalized the cardiac phenotype of SAP-IFN- γ mice (Figure 2) and significantly reduced cardiac infiltration by inflammatory cells (Figure 3), it was of great interest to investigate the expression levels of pro-inflammatory cytokines in the hearts of TNF- $\alpha^{-/-}$ SAP-IFN- γ double-mutant mice. In accord with the results of our previous study,¹⁵ the expression of the pro-inflammatory cytokines TNF- α , IL-12, MCP-1, and MIP-1 α was increased in TNF- $\alpha^{+/+}$ SAP-IFN- γ hearts (Figure 5) compared with TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ controls. Interestingly, the TNF- $\alpha^{-/-}$ SAP-IFN- γ hearts showed high-level expression of IL-12, MCP-1, and MIP-1 α , which was comparable to that of TNF- $\alpha^{+/+}$ SAP-IFN- γ controls. Thus, introduction of the homozygous TNF- α knockout defect did not lead to normalization of expression of pro-inflammatory cytokines in SAP-IFN- γ hearts.

Figure 4. Serum cTNT concentrations. Serum concentrations of cTNT of TNF- $\alpha^{+/+}$, TNF- $\alpha^{-/-}$, TNF- $\alpha^{+/+}$ SAP-IFN- γ , and TNF- $\alpha^{-/-}$ SAP-IFN- γ mice. Note the significant increase of cTNT serum concentrations of TNF- $\alpha^{+/+}$ SAP-IFN- γ mice compared with TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ controls, indicative of the cardiac phenotype of myocarditis and cardiomyopathy of such mice. The cTNT serum levels of the TNF- $\alpha^{-/-}$ SAP-IFN- γ mice were significantly reduced compared with the TNF- $\alpha^{+/+}$ SAP-IFN- γ mice, corroborating the normalization of cardiac phenotype of SAP-IFN- γ mice, as induced by TNF- α gene inactivation. Data were analyzed by analysis of variance. ns indicates not significant. ** $P < 0.01$, *** $P < 0.001$.

Figure 5. Cytokine expression. Total RNA was prepared from the hearts of male $TNF-\alpha^{+/+}$, $TNF-\alpha^{-/-}$, $TNF-\alpha^{+/+}$ SAP-IFN- γ , and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice, aged 6 months, and subjected to specific quantitative real-time RT-PCRs. The $2^{-\Delta\Delta CT}$ values of the $TNF-\alpha^{+/+}$ control mice were set to 100%. Cardiac expression of pro-inflammatory cytokines did not differ significantly between $TNF-\alpha^{+/+}$ and $TNF-\alpha^{-/-}$ control mice, and no $TNF-\alpha$ transcription was detectable in the hearts of $TNF-\alpha^{-/-}$ and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice. In accord with our previous report,¹⁵ the expression of the pro-inflammatory cytokines TNF- α , IL-12, MCP-1, and MIP-1 α was significantly increased in $TNF-\alpha^{+/+}$ SAP-IFN- γ hearts compared with $TNF-\alpha^{+/+}$ controls. Interestingly, IL-12, MCP-1, and MIP-1 α expression levels were also significantly increased in the $TNF-\alpha^{-/-}$ SAP-IFN- γ hearts compared with the $TNF-\alpha^{+/+}$ or $TNF-\alpha^{-/-}$ controls, and the values reached comparable (no significant differences) levels as the $TNF-\alpha^{+/+}$ SAP-IFN- γ mice. The values presented are the mean $\pm$ SD of eight animals per genotype. Data were analyzed by *t*-test (TNF- α) or analysis of variance (MCP-1, IL-12, and MIP-1 α). ns indicates not significant. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Restoration of Cardiac Function of SAP-IFN- γ Mice by TNF- α Inactivation

In our next experiment, we investigated whether the normalization of cardiac fine structure induced in SAP-IFN- γ hearts by TNF- α inactivation would also influence cardiac function. To this end, we investigated anesthetized $TNF-\alpha^{+/+}$ SAP-IFN- γ and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice and $TNF-\alpha^{+/+}$ and $TNF-\alpha^{-/-}$ controls at the ages of 6 and 9 months, respectively, by echocardiography. The results are presented as LVEDD and percentage FS, which indicates the relative cardiac ejection efficiency and, thus, represents an adequate marker of murine cardiac function. The LVEDD values of $TNF-\alpha^{+/+}$ SAP-IFN- γ hearts were increased (Figure 6), and percentage FS was decreased (Figure 7), compared with $TNF-\alpha^{+/+}$ or $TNF-\alpha^{-/-}$ controls. These findings are in full accord with the phenotype of dilated cardiomyopathy, as described for aged SAP-IFN- γ mice in our previous study.¹⁵ Interestingly, the imaging revealed no pathological LVEDD and FS values in $TNF-\alpha^{-/-}$ SAP-IFN- γ hearts, indicating the potential of TNF- α gene inactivation to normalize the cardiac function of SAP-IFN- γ mice.

Influence of TNF- α Gene Inactivation on Apoptosis

To investigate whether the phenotypic normalization of $TNF-\alpha^{-/-}$ SAP-IFN- γ hearts was associated with alterations of apoptosis, quantitative TUNEL stainings were performed. As shown in Figure 8, the cardiac apoptosis rate of $TNF-\alpha^{+/+}$ SAP-IFN- γ mice was comparable to that of $TNF-\alpha^{+/+}$ and $TNF-\alpha^{-/-}$ animals, which is in full accordance with our previous results¹⁵ of comparable levels of apoptotic cells and caspase 3 and 5 expression between SAP-IFN- γ and nontransgenic control mice. Furthermore, no significant differences of apoptotic cells/HPF could be detected between $TNF-\alpha^{+/+}$ SAP-IFN- γ and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice (Figure 8).

TNF- α Defect Fails to Normalize Chronic Active Hepatitis of SAP-IFN- γ Mice

In our next experiment, we investigated whether the normalization of inflammatory reactions, as induced in the hearts of SAP-IFN- γ mice by TNF- α deficiency, could also be observed in the liver parenchyma. In a first approach to answer this question, we analyzed serum ALT concentrations of $TNF-\alpha^{+/+}$, $TNF-\alpha^{-/-}$, $TNF-\alpha^{+/+}$ SAP-IFN- γ , and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice at the age of 6 months. In full accord with the phenotype of chronic active hepatitis, as developed by SAP-IFN- γ mice, serum ALT concentrations of $TNF-\alpha^{+/+}$ SAP-IFN- γ mice, were significantly increased compared with those of $TNF-\alpha^{+/+}$ and $TNF-\alpha^{-/-}$ negative control mice (Figure 9). However, ALT concentrations

Figure 6. Echocardiographic analysis of LVEDD. The LVEDD values of $TNF-\alpha^{+/+}$, $TNF-\alpha^{-/-}$, $TNF-\alpha^{+/+}$ SAP-IFN- γ , and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice, aged 9 months, were determined by echocardiography. The LVEDD values of $TNF-\alpha^{+/+}$ SAP-IFN- γ mice were significantly increased compared with the $TNF-\alpha^{-/-}$ controls. In contrast, $TNF-\alpha^{-/-}$ SAP-IFN- γ mice showed a normal LVEDD, indicating the normalization of cardiac function of SAP-IFN- γ mice by TNF- α gene inactivation. Data shown represent the mean $\pm$ SD values of three independent echocardiographic analyses of three or four mice per genotype. Data were analyzed by analysis of variance. ns indicates not significant. ** $P < 0.01$.

of the $TNF-\alpha^{-/-}$ SAP-IFN- γ mice combining SAP-IFN- γ transgenesis with the homozygous $TNF-\alpha$ knockout defect were comparable to those of the $TNF-\alpha^{+/+}$ SAP-IFN- γ mice, indicating that $TNF-\alpha$ gene inactivation had only a minor influence on chronic active hepatitis of SAP-IFN- γ transgenic mice.

To further investigate the liver-specific effects of $TNF-\alpha$ gene inactivation, infiltration by CD4⁺, CD11c⁺, and F4/80⁺ cells was investigated in $TNF-\alpha^{+/+}$, $TNF-\alpha^{-/-}$, $TNF-\alpha^{+/+}$ SAP-IFN- γ , and $TNF-\alpha^{-/-}$ SAP-IFN- γ livers by immunofluorescence. The $TNF-\alpha^{+/+}$ SAP-IFN- γ mice showed an intensive infiltration of their liver parenchyma by dendritic cells, macrophages, and T cells (data not shown). However, for all inflammatory cells investigated, the $TNF-\alpha^{-/-}$ SAP-IFN- γ livers showed a comparable extent of infiltration as in the $TNF-\alpha^{+/+}$ SAP-IFN- γ mice, indicating that $TNF-\alpha$ inactivation had no influence on chronic active hepatitis of SAP-IFN- γ transgenic mice.

Figure 7. Echocardiographic analysis of FS. $TNF-\alpha^{+/+}$, $TNF-\alpha^{-/-}$, $TNF-\alpha^{+/+}$ SAP-IFN- γ , and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice, aged 6 months (A) and 9 months (B), were investigated by echocardiography, and percentage FS was calculated. The trend of results was identical in both age groups tested. In full accord with our own previous study,¹⁵ FS of $TNF-\alpha^{+/+}$ SAP-IFN- γ mice overexpressing IFN- γ in the context of an intact $TNF-\alpha$ allele was significantly reduced compared with both control groups. In contrast, $TNF-\alpha^{-/-}$ SAP-IFN- γ mice combining IFN- γ overexpression with homozygous $TNF-\alpha$ deficiency showed a normalized FS that was comparable to that of the $TNF-\alpha^{+/+}$ and $TNF-\alpha^{-/-}$ control mice (no significant differences, with the exception of the P value of the 6-month-old $TNF-\alpha^{-/-}$ and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice). Data shown represent the mean $\pm$ SD values of three independent echocardiographic analyses of 8 mice (A) and 3 or 4 mice (B) per genotype. Data were analyzed by analysis of variance. ns indicates not significant. * $P < 0.05$, *** $P < 0.001$.

Figure 8. TUNEL staining. TUNEL staining was performed with cardiac slides of $TNF-\alpha^{+/+}$, $TNF-\alpha^{-/-}$, $TNF-\alpha^{+/+}$ SAP-IFN- γ , and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice. Data shown represent mean values of eight mice, aged 6 months, per genotype. Data were analyzed by analysis of variance. ns indicates not significant.

tivation had no influence on chronic active hepatitis of SAP-IFN- γ transgenic mice.

TNF- α Gene Inactivation Significantly Increases the Life Span of SAP-IFN- γ Mice

In our previous publication,¹⁵ we had speculated that the most plausible explanation for the premature death

Figure 9. Serum ALT concentrations. ALT concentrations were determined in the serum samples of $TNF-\alpha^{+/+}$, $TNF-\alpha^{-/-}$, $TNF-\alpha^{+/+}$ SAP-IFN- γ , and $TNF-\alpha^{-/-}$ SAP-IFN- γ mice, aged 6 months. As to be expected, ALT concentrations of $TNF-\alpha^{+/+}$ and $TNF-\alpha^{-/-}$ control mice were in a normal range. In accord with their phenotype of chronic active hepatitis,¹⁶ the mean ALT level of $TNF-\alpha^{+/+}$ SAP-IFN- γ mice was significantly increased compared with that of the $TNF-\alpha^{-/-}$ and $TNF-\alpha^{+/+}$ controls. The ALT concentrations of the $TNF-\alpha^{-/-}$ SAP-IFN- γ mice were comparable to those of the $TNF-\alpha^{+/+}$ SAP-IFN- γ mice, indicating that $TNF-\alpha$ gene inactivation had no significant influence on chronic active hepatitis, as developed by SAP-IFN- γ transgenic mice. Data shown represent the mean $\pm$ SD values of 10 animals per genotype. Data were analyzed by analysis of variance. ns indicates not significant. * $P < 0.05$, ** $P < 0.01$.

of *SAP-IFN- γ* mice is heart failure. Given the normalization of cardiac morphological features and function as inducible in these IFN- γ -overexpressing mice by the introduction of the *TNF- α* gene defect, we speculated that the constitutive *TNF- α* gene inactivation might also increase the life span of *SAP-IFN- γ* mice. To answer this question, we generated a long-term survival study and Kaplan-Meier plots. As shown in Figure 1, virtually all *TNF- α ^{+/-} SAP-IFN- γ* mice died within an age span of 6 to 12 months, which is in full accord with the data previously described for *SAP-IFN- γ* mice.^{15,16} In contrast, the life span of *TNF- α ^{-/-} SAP-IFN- γ* mice was significantly increased, clearly indicating the beneficial effect of *TNF- α* gene inactivation on *SAP-IFN- γ* life expectancy.

Discussion

In a recently published study,¹⁵ we showed that transgenic mice with constitutively elevated IFN- γ serum levels develop chronic active myocarditis and cardiomyopathy and, thus, reveal a hitherto unknown IFN- γ -mediated cardiac pathomechanism. To investigate the pathomechanism underlying myocarditis and cardiomyopathy occurring in IFN- γ -overexpressing *SAP-IFN- γ* mice, we conducted a follow-up study to analyze the cardiac phenotype of *Rag1^{-/-} SAP-IFN- γ* and *TNF- α ^{-/-} SAP-IFN- γ* double-mutant mice combining transgenic IFN- γ overexpression with a lack of adaptive immunity and *TNF- α* deficiency, respectively.

T- and B-cell-deficient *Rag1^{-/-} SAP-IFN- γ* mice showed intensive cardiac inflammatory reactions and a dramatic impairment of life span, indicating that IFN- γ -mediated myocarditis is not mediated by (auto)immune responses. On the contrary, these data provide unequivocal evidence that the immune system has a protective effect on the phenotype of *SAP-IFN- γ* mice; we are currently conducting experiments to elucidate the underlying mechanism.

We found that the *TNF- α* knockout defect had the potential to normalize the cardiac phenotype and function of *SAP-IFN- γ* mice. In particular, the introduction of the *TNF- α* knockout defect virtually completely inhibited infiltration of *SAP-IFN- γ* hearts by F4/80⁺ macrophages and significantly reduced infiltration by CD11c⁺ dendritic cells. In contrast, the cardiac density of CD4⁺ T cells was not significantly affected by the *TNF- α* knockout. Because T cells represent strong producers of MCP-1 and MIP-1 α ,²⁶⁻²⁸ it was not surprising that cardiac synthesis of these pro-inflammatory cytokines was unaffected in *TNF- α ^{-/-} SAP-IFN- γ* hearts. In this context it has to be mentioned that MCP-1, MIP-1 α , and IL-12 are also synthesized by cardiomyocytes and endothelial cells²⁹⁻³¹ and dendritic cells,³² which could have further contributed to the similarity of cytokine transcription, as observed between *TNF- α* intact and defect *SAP-IFN- γ* mice. Consistent with the structural and immunological findings, we observed a virtual normalization of FS of *SAP-IFN- γ* hearts induced by *TNF- α* gene deficiency, indicative of the *TNF- α* dependency of the cardiac phenotype of *SAP-IFN- γ* mice. The latter finding was associated with a significant increase of life span

of *TNF- α ^{-/-} SAP-IFN- γ* mice, challenging the suggestion of Toyonaga and coworkers¹⁶ that the premature death of *SAP-IFN- γ* mice is induced by infections with Gram-negative bacteria and corroborating our previous hypothesis that the life span of such animals is determined by their phenotype of CHF.¹⁵

There are two interesting results in the current study that need special attention and discussion. First, the number of cardiac macrophages was considerably reduced in *TNF- α ^{-/-} SAP-IFN- γ* mice compared with *TNF- α ^{+/-} SAP-IFN- γ* controls; although both strains had a similar expression level of MIP-1 α and MCP-1. Obviously, the high-level MIP-1 α and MCP-1 expression, as found in the *TNF- α ^{-/-} SAP-IFN- γ* hearts, did not have the potential of attracting macrophages into the cardiac tissue. This finding is a bit surprising at first glance, because macrophage recruitment represents a pivotal function of the MIP-1 α and MCP-1 chemokines.^{33,34} However, macrophages are the major producers of *TNF- α* and, interestingly, are also highly responsive to this cytokine³⁵; multiple functions of macrophages depend on paracrine or autocrine *TNF- α* signaling.³⁶⁻³⁸ Thus, the most plausible explanation for the impaired infiltration of *TNF- α ^{-/-} SAP-IFN- γ* hearts by macrophages is that the cytokine *TNF- α* is also necessary to allow macrophages to adequately respond to MIP-1 α and MCP-1.

Second, *TNF- α* inactivation had no influence on the hepatitis phenotype developed by *SAP-IFN- γ* transgenic mice. However, these results are in accord with a previously published animal model of concanavalin A-induced hepatitis,³⁹ in which hepatitis was suppressed in *IFN- γ ^{-/-}* mice but not in *TNF- α ^{-/-}* mice, showing that at least this type of experimental hepatitis is mediated by IFN- γ but not by *TNF- α* .

Our previous study¹⁵ demonstrated a direct cardiotoxic effect of the pro-inflammatory cytokine IFN- γ . The current work was aimed to shed light on the pathomechanism by which IFN- γ can trigger myocarditis and cardiomyopathy in *SAP-IFN- γ* mice. We could exclude that adaptive immunity contributes to myocarditis of *SAP-IFN- γ* mice. However, we could clearly show that the detrimental IFN- γ effects observed in the IFN- γ -overexpressing animals were almost exclusively mediated by *TNF- α* . The latter result is in accord with a multitude of experimental data indicating cardiotoxic properties of *TNF- α* .^{10,11,40} The cardiotoxic potential of this cytokine is also indirectly supported by clinical data, showing increased serum and cardiac *TNF- α* levels during myocarditis and CHF.^{5-8,41} Recent publications⁴² have shown that *TNF- α* can activate pro- and anti-apoptotic pathways in cardiomyocytes. However, we could not find significantly modified apoptosis rates or caspase expression levels in the *SAP-IFN- γ* mice. We are currently planning studies to show the precise sources of *TNF- α* synthesis in *SAP-IFN- γ* hearts and to clarify the pathomechanism underlying *TNF- α* cardiotoxicity. Because *TNF- α* -mediated mechanisms of cardiotoxicity are most likely also operative in human patients, we further want to use the *SAP-IFN- γ* model to test new therapeutic approaches of *TNF- α* inhibition.

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