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Chronic Inflammatory Cardiomyopathy of Interferon \(\gamma\)-Overexpressing Transgenic Mice Is Mediated by Tumor Necrosis Factor-\(\alpha\)

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We recently described a model of inflammatory cardiomyopathy in interferon (IFN)-\(\gamma\) overexpressing transgenic mice stably circulating IFN-\(\gamma\) in the serum referred to as SAP–IFN-\(\gamma\) mice. SAP–IFN-\(\gamma\) transgenic mice show cardiatic 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-\(\gamma\) cardiomyopathy might be mediated by (auto)immune processes or tumor necrosis factor (TNF)-\(\alpha\) synthesis from IFN-\(\gamma\)-activated macrophages. To verify these hypotheses, we crossed SAP–IFN-\(\gamma\) transgenic mice with immunodeficient Rag1\(^{-/-}\) or TNF-\(\alpha\)\(^{-/-}\) knockout mice and analyzed the cardiac phenotype of the resulting double-mutant offspring. Immunodeficient Rag1\(^{-/-}\) SAP–IFN-\(\gamma\) mice had a decreased impaired life span and intensive cardiac inflammatory reactions, showing that the cardiotoxic IFN-\(\gamma\) effect operative in SAP–IFN-\(\gamma\) mice was not mediated by an adaptive immune mechanism. SAP–IFN-\(\gamma\) 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-\(\gamma\) transgenic controls. Thus, myocarditis and cardiomyopathy developing in IFN-\(\gamma\)-overexpressing transgenic mice is, to a significant degree, mediated by TNF-\(\alpha\). TNF-\(\alpha\)-mediated cardiotoxicity in SAP–IFN-\(\gamma\) 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)-\(\alpha\), IL-1\(\beta\), 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-\(\alpha\) 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-\(\alpha\) 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)-\(\gamma\) represents another cytokine with pleiotropic pro-inflammatory effects. Although this cytokine is a potent inducer of TNF-\(\alpha\) synthesis,14 there is only

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analyses revealed that the role of IFN-γ in chronic heart disease, we recently published a transgenic mouse model of IFN-γ-mediated cardiomyopathy.\textsuperscript{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.\textsuperscript{16} SAP–IFN-γ mice have developed chronic active hepatitis and myocarditis progressing to cardiomyopathy.\textsuperscript{15,16} The cardiac phenotype of SAP–IFN-γ mice was characterized by progressive infiltrations with CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and Mac2\textsuperscript{+} macrophages and high-level monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, 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\textsuperscript{-/-} animals. The resulting Rag1 SAP–IFN-γ mice exhibited cardiac inflammatory reactions and showed a dramatic impairment of life expectancy compared with immunocompetent Rag1\textsuperscript{+/-} 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,\textsuperscript{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\textsuperscript{-/-} knockout mice\textsuperscript{18} 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), because the LVEDD and FS were reduced in the double-mutant mice compared with immunocompetent TNF-α\textsuperscript{+/-} SAP–IFN-γ experimental mice. 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\textsubscript{5} (official strain designation Tg(APCS-lng)Smeg, hereafter referred to as SAP–IFN-γ) have been previously described.\textsuperscript{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.\textsuperscript{19} Rag1\textsuperscript{+/-} SAP–IFN-γ and Rag1\textsuperscript{-/-} experimental animals were generated by crossing Rag1\textsuperscript{+/-} SAP–IFN-γ males and Rag1\textsuperscript{-/-} females (official strain designation: B6.129S7-Rag1\textsubscript{tm1Mom}; The Jackson Laboratory, Bar Harbor, ME). Rag1\textsuperscript{+/-} SAP–IFN-γ and Rag1\textsuperscript{-/-} mice were bred by mating SAP–IFN-γ and Rag1\textsuperscript{-/-} mice.

We further established a cross of TNF-α\textsuperscript{+/-} SAP–IFN-γ males and TNF-α\textsuperscript{-/-} females (official strain designation: B6.129S-Tnf\textsubscript{<tm1Gki>}, stock 005540 The Jackson Laboratory) to obtain TNF-α\textsuperscript{-/-} SAP–IFN-γ and TNF-α\textsuperscript{-/-} experimental animals. TNF-α\textsuperscript{-/-} SAP–IFN-γ and TNF-α\textsuperscript{-/-} experimental animals were generated by mating SAP–IFN-γ and TNF-α\textsuperscript{-/-} mice.

Transgenic offspring could be discriminated from their nontransgenic littermates by PCR amplification, as previously described.\textsuperscript{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 CO\textsubscript{2}, and cardiac tissue specimens were fixed in 4% buffered formaldehyde solution. Sections were immediately embedded in paraffin, and sections (4 μm 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 Hoechst33342 (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 Reflotet 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). 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. A one-step RT-PCR was performed in 25-μL reactions in a 96-well spectrophotometric 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’-TACTCCGAG-CGGTTCACTGTGTTCA-3’; antisense, 5’-CAGCTGCTGCTGTTCA-3’; probe, 5’-TCTTCTCATTCCTGCTTGTGG-3’), MCP-1 (sense, 5’-CTCCTGGGCTGTCCTGTTCA-3’; antisense, 5’-CCAGCTACTTACATTGG-GATC-3’; probe, 5’-CTCAGCCAGATGCAGTTAACGCCC-3’), cTNT (sense, 5’-CTGCAAACCACTTCTCAGC-3’; antisense, 5’-CAGCTCCTCAAGCTCTAGG-3’; probe, 5’-ACTGCTGGTCTGCTCTCTACAGGC-3’), TNF-α (sense, 5’-CTGAGGAGGCCATTTG-3’; antisense, 5’-ATGAGGAGGAGCCATTTG-3’; probe, 5’-TACCTGGATCCGGGGTACGGTCC-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’-ACCACTCCCAATGATATTGGAG-3’; antisense, 5’-ATGCTCATACTTGCTCACACAGGAGG-3’; probe, 5’-CTGCGACTTGGCTGCGGAA-3’). TaqMan hybridization probes were double labeled with 6-carboxyfluorescein and carboxy tetramethyl-rhodamine as quencher. All primers with 6-carboxyfluorescein as reporter fluorophore and 6-carboxy tetramethyl-rhodamine as quencher. All primers with 6-carboxyfluorescein as reporter fluorophore and 6-carboxy tetramethyl-rhodamine as quencher.

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Sample. The 2^(−ΔΔCT) values of the TNF-α^+/− mice for each mRNA analyzed were set to 100%.

**Mouse Echocardiography**

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

**Statistical Analysis**

All data are presented as mean ± 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–INF–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 have been detected in the hearts of SAP–INF–γ mutants, 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–INF–γ transgenic strain was crossed to the Rag1^−/− immunodeficient knockout variant that completely lacks T and B cells. The resulting Rag1^−/− SAP–INF–γ double-mutant mice and their Rag1^+/− SAP–INF–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–INF–γ mice died within the age span of 6 and 12 months, which is in full accord with the data previously described for SAP–INF–γ mice.15,16 To our surprise, the life span of Rag1^−/− SAP–INF–γ mice was significantly impaired, clearly indicating that the adaptive immune system has a beneficial effect on SAP–INF–γ life expectancy and excluding the possibility that myocarditis of SAP–INF–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–INF–γ mice (data not shown), suggesting that the cardiotoxic IFN–γ effect, as observed in SAP–INF–γ mice, was not mediated by an adaptive immune mechanism.

**Normalization of Cardiac Phenotype and cTNT Levels of SAP–INF–γ Mice by TNF–α Inactivation**

To further investigate the pathomechanisms leading to myocarditis and cardiomyopathy in IFN–γ-overexpressing SAP–INF–γ mice, the SAP–INF–γ transgenic strain...
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 work16 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 macrophages 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
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...reactions could be found in the hearts of the nontransgenic TNF-

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Cardiac Pathomechanism of SAP–IFN-γ Mice

...gene deficiency, were still increased compared with those of the TNF-α/−/− and

...Serum cTNT concentrations. Serum concentrations of cTNT of

...TNF-α/−/− SAP–IFN-γ mice were significantly increased compared with those of the TNF-α/−/− and

...characteristic of myocarditis of SAP–IFN-γ hearts. The anti-inflammatory effect was statistically significant

...cardiac phenotype of SAP–IFN-γ mice, as mediated by TNF-α gene inactivation.

Phenotypic Normalization of TNF-α/−/− 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-1α, 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-α/−/− 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-1α was increased in TNF-α/−/− SAP–IFN-γ hearts (Figure 5) compared with TNF-α/−/− and TNF-α/−/− controls. Interestingly, the TNF-α/−/− SAP–IFN-γ hearts showed high-level expression of IL-12, MCP-1, and MIP-1α, which was comparable to that of TNF-α/−/− 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.

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Influence of TNF-α Gene Inactivation on Apoptosis

To investigate whether the phenotypic normalization of TNF-α−/− 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-α+/− SAP–IFN-γ mice was comparable to that of TNF-α+/− and TNF-α−/− animals, which is in full accordance with our previous results10 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-α−/− SAP–IFN-γ and TNF-α+/− 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-α+/−, TNF-α−/−, TNF-α−/− SAP–IFN-γ, and TNF-α+/− 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-α−/− SAP–IFN-γ mice, were significantly increased compared with those of TNF-α+/− and TNF-α−/− negative control mice (Figure 9). However, ALT concentrations

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-α−/− SAP–IFN-γ and TNF-α−/− SAP–IFN-γ mice and TNF-α+/− and TNF-α−/− 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-α−/− SAP–IFN-γ hearts were increased (Figure 6), and percentage FS was decreased (Figure 7), compared with TNF-α+/− or TNF-α−/− 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-α−/− SAP–IFN-γ hearts, indicating the potential of TNF-α gene inactivation to normalize the cardiac function of SAP–IFN-γ mice.
of the TNF-α−/− SAP–IFN-γ mice combining SAP–IFN-γ transgenesis with the homozygous TNF-α knockout defect were comparable to those of the TNF-α+/− SAP–IFN-γ mice, indicating that TNF-α 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-α gene inactivation, infiltration by CD4+, CD11c+, and F4/80+ cells was investigated in TNF-α+/−, TNF-α−/−, SAP–IFN-γ, and TNF-α−/− SAP–IFN-γ livers by immunofluorescence. The TNF-α−/− 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-α−/− SAP–IFN-γ livers showed a comparable extent of infiltration as in the TNF-α+/− SAP–IFN-γ mice, indicating that TNF-α inactivation had no influence on chronic active hepatitis of SAP–IFN-γ transgenic mice.

Figure 8. TUNEL staining. TUNEL staining was performed with cardiac slides of TNF-α−/−, TNF-α+/−, TNFα+/− SAP–IFN-γ, and TNF-α−/− 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.

Figure 7. Echocardiographic analysis of FS. TNF-α+/−, TNF-α−/−, TNF-α+/− SAP–IFN-γ, and TNF-α+/− 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,7 FS of TNF-α−/− SAP–IFN-γ mice overexpressing IFN-γ in the context of an intact TNFα allele was significantly reduced compared with both control groups. In contrast, TNF-α−/− SAP–IFN-γ mice combining IFN-γ overexpression with homozygous TNF-α deficiency showed a normalized FS that was comparable to that of the TNF-α−/− and TNF-α+/− control mice (no significant differences, with the exception of the P value of the 6-month-old TNF-α−/− and TNF-α+/− SAP–IFN-γ mice). Data shown represent the mean ± 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 < 0.01.

Figure 9. Serum ALT concentrations. ALT concentrations were determined in the serum samples of TNF-α+/−, TNF-α−/−, TNF-α+/− SAP–IFN-γ, and TNF-α−/− SAP–IFN-γ mice, aged 6 months. As to be expected, ALT concentrations of TNF-α+/− and TNF-α−/− control mice were in a normal range. In accord with their phenotype of chronic active hepatitis,10 the mean ALT level of TNF-α+/− SAP–IFN-γ mice was significantly increased compared with that of the TNF-α−/− and TNF-α+/− controls. The ALT concentrations of the TNF-α−/− SAP–IFN-γ mice were comparable to those of the TNF-α+/− SAP–IFN-γ mice, indicating that TNF-α gene inactivation had no significant influence on chronic active hepatitis, as developed by SAP–IFN-γ transgenic mice. Data shown represent the mean ± 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 MIP-1α and MIP-1β,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, IL-4, and IL-12 are also synthesized by cardiomycocytes and endothelial cells39–41 and dendritic cells,42 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-1α and MCP-1. Obviously, the high-level MIP-1α 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-1α 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-1α 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 cardio- toxic 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.10,11,14,40 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 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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