Impaired up-regulation of CD25 on CD4\(^+\) T cells in IFN-\(\gamma\) knockout mice is associated with progression of myocarditis to heart failure


Departments of *Pathology, †Medicine, and §Comparative Medicine and the ¶W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins Medical Institutions, Baltimore, MD 21205

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Inflammation has been recognized increasingly as a critical pathologic component of a number of heart diseases. A mouse model of autoimmune myocarditis was developed to study the role of immune mediators in the development of cardiac dysfunction. We have found previously that IFN-\(\gamma\) deficiency promotes inflammation in murine myocarditis. It has been unclear, however, how IFN-\(\gamma\) deficiency in myocarditis affects cardiac function and what underlying immune mechanisms are responsible for these effects. In this work, we show that IFN-\(\gamma\) knockout (KO) mice have more pronounced systolic and diastolic dysfunction and greater frequency of progression to dilated cardiomyopathy and heart failure compared with WT mice. Cardiac dysfunction in the KO mice is associated with the expansion of activated (CD4\(^{4+}\)) CD3\(^+\) T cells due to reduced apoptosis of CD4\(^{+}\), but not CD8\(^{+}\), T cells. CD4\(^{+}\) T cells in the KO mice showed impaired up-regulation of CD25 upon activation, resulting in the expansion of CD4\(^{+}\)CD44\(^{+}\)CD25\(^{-}\) T cells and their infiltration into the heart. CD4\(^{+}\)CD25\(^{-}\) T cells are less apoptosis-prone compared with the CD25\(^+\) population, and their infiltration into the heart is associated with greater severity of myocarditis. We conclude that IFN-\(\gamma\)-deficiency in autoimmune myocarditis is associated with preferential expansion of CD4\(^{+}\)CD44\(^{+}\)CD25\(^{-}\) T cells resulting in increased cardiac inflammation. An exaggerated inflammatory response in IFN-\(\gamma\)-KO mice causes cardiac dysfunction, leading to dilated cardiomyopathy and heart failure.

Inflammation and autoimmunity increasingly have been recognized as important pathogenic components in cardiac diseases and the development of cardiac dysfunction. A number of cytokines, including IL-6 and tumor necrosis factor \(\alpha\), have been shown to contribute to the progression of heart failure. In inflammatory heart diseases, cytokines can affect disease outcomes by regulating the degree of inflammation, by mediating cardiac remodeling through their effects on fibrosis and cardiomyocyte hypertrophy, or by directly influencing cardiomyocyte function.

IFN-\(\gamma\), a prototypic T helper 1 cytokine, mainly produced by natural killer and T cells, stimulates T helper 1 T cell development, activates macrophages, induces MHC class II expression, promotes delayed-type hypersensitivity reactions, plays an important role in clearing intracellular bacteria and parasites, and exhibits antiviral activity (1). Despite its reported proinflammatory activity (2–4), IFN-\(\gamma\) has been shown to suppress inflammation in many animal models of autoimmune disease (5–7). It has been demonstrated previously that IFN-\(\gamma\) suppresses acute inflammation and that IFN-\(\gamma\)-deficient mice develop an exaggerated inflammatory response in a model of cardiac myosin (CM)-induced experimental autoimmune myocarditis (EAM) (8–12).

It has been unclear, however, how IFN-\(\gamma\) deficiency in EAM affects cardiac function. Exaggerated acute myocardial inflammation in IFN-\(\gamma\)-knockout (KO) mice may lead to cardiomyocyte damage and adverse cardiac remodeling, promoting heart failure. At the same time, IFN-\(\gamma\) has been reported to exert direct negative inotropic effects on cardiomyocytes, possibly through activation of inducible nitric oxide synthase (13, 14). Based on the latter observation, the prediction can be made that IFN-\(\gamma\) deficiency is beneficial in a setting of cardiac inflammation. Furthermore, it is important to know how IFN-\(\gamma\) deficiency affects the composition of the inflammatory myocardial infiltrate and to identify the underlying immune mechanisms for these changes, because the quality as well as the quantity of the local inflammatory response can determine in vivo organ damage. To address these questions, we studied temporal progression of cardiac dysfunction as well as associated systemic and local cardiac immune phenomena in IFN-\(\gamma\)-KO mice as compared with WT mice.

Methods

Mice. EAM was induced in 10- to 12-week-old female WT and IFN-\(\gamma\)-KO BALB/c mice obtained from The Jackson Laboratory and maintained in The Johns Hopkins University School of Medicine conventional animal facility. The animal work was approved by the Animal Care and Use Committee of The Johns Hopkins University.

Induction and Assessment of EAM. CM immunization and histologic assessment were performed as described in ref. 11. Gross scores were based on the extent of white discoloration on a scale from 0 to 5 as described for histologic scores.

Assessment of Cardiac Function. Pressure–volume studies were performed as described in ref. 15. The time constant of isovolumetric relaxation (\(\tau\)) was derived by using an assumption of monoeponential pressure decay with a nonzero asymptote (16). Transthoracic echocardiography was performed as described in refs. 17 and 18.

In Vitro Stimulation. Spleen and peripheral blood samples were treated for 5 min with ACK lysing buffer (pH 7.4) (BioWhittaker). Live cells were counted by trypan blue exclusion. Cells were cultured in 24-well plates at an initial cell density of 5 \(\times\) 10\(^7\) cells per ml in complete RPMI medium 1640 (Life Technologies, Grand Island, NY) with the additional supplementation described in ref. 19. In the presence of 10 \(\mu\)g/ml anti-CD3 mAb or 10 \(\mu\)g/ml anti-CD3 and 5 \(\mu\)g/ml anti-CD28 for 12 h or in the

Abbreviations: CM, cardiac myosin; EAM, experimental autoimmune myocarditis; KO, knockout; DCM, dilated cardiomyopathy; 7AAD, 7-aminoactinomycin D.

1M.A. and D.G. contributed equally to this work.

To whom correspondence should be addressed at: Department of Pathology, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Ross Building, Room 659, Baltimore, MD 21205. E-mail: nrrose@jhsp.edu.

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presence of 10 μg/ml CM for 3 days. Peripheral blood cells were stimulated for 4 h with 50 ng/ml phorbol 12-myristate 13-acetate (Life Technologies) and 1 μmol/liter ionomycin (Sigma).

Flow Cytometry. Mouse hearts were digested, and flow cytometry was performed as described in refs. 20 and 21. CD25+ cells were detected with allophycocyanin-labeled Ab (clone PC-61, Pharmingen). Cells were fixed overnight with Cytofix (pH 7.4) (Pharmingen). Annexin V and 7-aminoactinomycin D (7AAD) stains and assessment of heart cells were performed on unfixed cells.

Statistical Analyses. The Mann–Whitney U test was used to compare myocarditis scores, and Student’s t test was used for other comparisons. Associations between cellular populations in the heart were assessed using linear regression. The data were analyzed by using SIGMASTAT 3.01 software (SPSS, Chicago). P ≤ 0.05 was considered statistically significant.

Results

Increased Cardiac Pathology in IFN-γ KO Mice. We found that the KO mice had significantly higher gross and histologic scores of disease not only during the acute phase of disease (days 21–24) but also at later time points, including days 31–33, 42–44, and 58–62 (chronic phase) after immunization (Fig. 5 a and b, which is published as supporting information on the PNAS web site). On microscopic examination, the KO mice exhibited more intense inflammatory infiltration, especially during the acute phase, and greater cardiac fibrosis, which was more pronounced at later time points. Flow-cytometric examination of the heart infiltrates in the WT mice revealed that inflammation (assessed by percentage of infiltrating CD45+ leukocytes) peaked by day 15 (Fig. 5c). In contrast, peak inflammation in the KO mice occurred on days 21–24 and, at this time point, was associated with significantly higher percentages of infiltrating leukocytes compared with the WT mice. At later times, myocardial inflammation declined and did not differ significantly between the two groups.

Effects of IFN-γ Deficiency on Cardiac Function. Increased myocarditis scores in IFN-γ KO mice were accompanied by increases in heart weight (data not shown) and the heart-weight/body-weight ratio indicative of the development of cardiomyopathy (Fig. 1 a).

To study how IFN-γ deficiency affects cardiac function in EAM, we performed in vivo measurements of left-ventricular function. We found greater impairment of systolic and diastolic function in the KO compared with the WT mice (Fig. 1 b–f). This impairment was most pronounced during the acute phase of disease (days 21–24) with indices of cardiac function improving by days 42–44. Day 58–62 measurements, however, were indicative of further decompensation in the KO mice associated with the second peak of increase in heart-weight/body-weight ratios. Impaired cardiac performance, including such parameters as cardiac output, maximal rate of pressure development (dP/dtmax), time constant of pressure decay (τ), and end-diastolic elastance (Eed) normalized to heart weight, which represents passive stiffness, were obtained by using the in vivo pressure-volume method. Data are presented as mean ± SE. Day 0, n = 6 (WT) and n = 4 (KO); days 21–24, n = 9 in both groups; days 31–33, n = 12 in both groups; days 42–44, n = 9 (WT) and n = 8 (KO); days 58–62, n = 6 in both groups. * P < 0.05, compared with WT mice during the same time point.

Fig. 1. IFN-γ deficiency in EAM is associated with greater cardiac dysfunction. Shown are heart-weight/body-weight ratio (HW/BW) (a); cardiac output (CO) (b); maximal rate of pressure development (dP/dtmax) (c); stroke work-end-diastolic volume relations, or preload-recruitable stroke work (PRSW) (d); time constant of isovolumic pressure decay (τ) (e); and end-diastolic elastance (Eed) normalized to heart weight, which represents passive stiffness (f) in WT (●) and IFN-γ KO (○) mice. Day 0 represents unimmunized age-matched controls. Data in b–f were obtained by using the in vivo pressure-volume method. Data are presented as mean ± SE. Day 0, n = 6 (WT) and n = 4 (KO); days 21–24, n = 9 in both groups; days 31–33, n = 12 in both groups; days 42–44, n = 9 (WT) and n = 8 (KO); days 58–62, n = 6 in both groups. * P < 0.05, compared with WT mice during the same time point.
secondary pneumonia and “heart failure cells” (Fig. 2 k and l). DCM was observed starting day 23 after immunization, and its severity, as assessed by end-diastolic volume, significantly correlated with the degree of myocardial fibrosis (data not shown).

**IFN-γ Deficiency Leads to Reduced Apoptosis and Systemic Expansion of Activated T Cells.** To assess the immune mechanisms of enhanced disease in IFN-γ KO mice, we first studied the peripheral blood T cell population after CM immunization. The KO mice had significantly greater percentages of CD3+ T cells on day 16 after immunization (see Table 1, which is published as supporting information on the PNAS web site). Percentages of activated CD3+ T cells expressing high levels of CD44 (CD44high) were increased significantly in the KO mice starting on day 3 after immunization with the absolute difference between the two groups being the greatest on day 16 (Table 1). Interestingly, in the KO mice this population of activated T cells expanded up to day 16, whereas in the WT mice these cells stopped expanding around day 9. To test the hypothesis that the greater expansion of activated T cells in the KO mice is associated with reduced activation-induced cell death, we assessed the number of T cells undergoing early apoptosis (annexin V+7AAD−). We found that the KO mice had a significantly lower percentage of CD3+ T cells undergoing early apoptosis on day 9 after immunization (Table 1) than the WT mice. Consistent with the observations in peripheral blood, IFN-γ KO mice had increased proportions of CD3+ T cells in the acute heart infiltrate (data not shown), and these T cells had greater intensity of CD44 expression (Fig. 3 a and b), indicating the presence of not only quantitative but also qualitative differences in the heart infiltrate compared with the WT mice.

**CD4+ vs. CD8+ T Cell Subsets in EAM in IFN-γ Deficiency.** It has been shown previously that IFN-γ controls the expansion of both CD4+ and CD8+ T cells in response to immune activation (22–24). We reported previously that IFN-γ deficiency in EAM leads to expansion of both subsets in the spleen (11). It has been unclear, however, which subset is more important in promoting disease in IFN-γ KO mice. To investigate the role of CD4+ vs. CD8+ T cells, we assessed the degree of apoptosis in both subsets in lymph nodes and spleen. CD4+, but not CD8+, T cells in IFN-γ KO mice consistently showed less apoptosis compared with T cells from WT mice (Fig. 3 c and d). To study a more disease-relevant population of T cells, we used flow cytometry on heart-derived cells that were enriched for leukocytes through depletion of cardiomyocytes (21). We found that CD4+ T cells predominated over CD8+ T cells in the heart infiltrate in both genotypes at all time points after immunization (data not shown). During the acute phase, there was an increased influx of both CD4+ and CD8+ T cells in the heart in IFN-γ KO mice compared with WT mice (Fig. 3 e and f). However, only the proportion of CD4+, but not CD8+, T cells of total infiltrating leukocytes was significantly increased in IFN-γ KO mice (Fig. 3 g and h). During the acute phase of EAM, IFN-γ KO mice had significantly increased CD4/CD8 ratios in the myocardium compared with WT mice (5.2 ± 3.3 in KO vs. 1.7 ± 0.9 in WT, P = 0.004).

**IFN-γ KO Mice Have Decreased Expression of CD25 on CD4+ T Cells in EAM.** To further characterize the pathologically relevant subset of CD4+ T cells, we looked at the expression of CD25, the α-subunit of the IL-2 receptor, on their surface. Expression of CD25 on CD4+ T cells trafficking through normal hearts of
unimmunized mice was similar between the KO and WT (Fig. 4a). The acute phase of EAM, however, was associated with a decreased proportion of CD4+/CD25+ T cells in the heart in IFN-γ KO mice compared with either immunized WT or unimmunized KO mice (Fig. 4a). Importantly, more severe myocardial inflammation, as assessed by percentages of CD45+/T cells in the heart, correlated with reduced proportions of CD4+/CD25+ T cells infiltrating the heart (Fig. 4b). There was no difference in CD25 expression on CD4+ T cells in the peripheral blood between the two genotypes at baseline (data not shown) or on days 3 and 16 after immunization. IFN-γ KO mice, however, had a significantly smaller percentage of CD4+/CD25+ T cells in the peripheral blood on day 9 after immunization (Fig. 4c) compared with WT mice. This time point was associated with an increase in CD25 expression compared with the baseline in WT mice, but this up-regulation was not observed in KO mice. In EAM, day 9 generally corresponded to the peak activation of lymphocytes in the periphery in response to the second injection of CM on day 7.

These findings led us to postulate that IFN-γ deficiency in EAM is associated with the impairment of activation-induced up-regulation of CD25 on CD4+ T cells. This hypothesis was supported by our observation that in vitro stimulation with either 12-myristate 13-acetate plus ionomycin or CM resulted in a lower CD25 expression on CD4+ T cells in IFN-γ KO mice (Fig. 4d and e). In vitro stimulation of splenocytes with either anti-CD3 or anti-CD28 mAbs resulted in up-regulation of CD25 on CD4+ T cells in both groups, but the percentage of CD25hi cells of total CD4+ T cells was consistently lower in the KO mice (Fig. 4f). This activation also resulted in a significantly greater percentage of CD4+/CD25hi T cells in IFN-γ KO mice compared with WT mice (data not shown). To gain greater insight into how the impaired up-regulation of CD25 on CD4+ T cells might be related to reduced apoptosis in IFN-γ KO mice, we looked at the degree of apoptosis in CD25+ T cell populations of CD4+ T cells from peripheral blood, lymph nodes, and spleen of immunized mice. We found that CD4+CD25+ T cells were consistently more death-prone than the CD4+CD25+ T cell population regardless of the genotype (Fig. 4g). Finally, we found that CD4+ T cells collected from immunized mice and treated with anti-CD3 and anti-CD28 mAbs that expressed high levels of CD25 had higher percentages of dead cells compared with those with intermediate levels of CD25 (Fig. 4h and i).

Discussion
Our results demonstrate that IFN-γ deficiency intensifies disease in EAM, producing an enhanced proinflammatory response that leads to greater cardiac damage and dysfunction. Increased myocardial inflammation in the absence of IFN-γ was associated with reduced apoptosis and expansion of activated T cells. The reduction in apoptosis was characteristic of the CD4+, but not the CD8+, T cell subset, and CD4+ T cells were predominant in the enhanced myocardial lesions. The importance of CD4+ T
cells in WT mice in EAM has been demonstrated by Smith and Allen (25) in experiments involving CD4⁺ T cell depletion and adoptive transfer. Importantly, we have found that percentages of CD4⁺ T cells, but not the total numbers of infiltrating leukocytes, in the heart infiltrate in WT mice significantly correlated with the degree of systolic dysfunction and progression to DCM during the chronic phase of EAM (21). In this work, we demonstrate that increased percentages in CD4⁺ T cells in the heart are associated with greater cardiac dysfunction in IFN-γ KO mice.

To our knowledge, this study is the first to demonstrate that IFN-γ deficiency in autoimmune disease is associated with reduced proportions of CD4⁺ T cells expressing CD25. CD4⁺CD25⁺ T cells have been shown to suppress autoaggressive T cell clones and, therefore, are critical in mediating peripheral tolerance (26, 27). Mice lacking CD25 develop lymphoproliferation and spontaneous autoimmune diseases, and human CD25 deficiency is associated with the occurrence of autoreactive T cells (28, 29). Classic regulatory CD4⁺CD25⁺ T cells develop in the thymus and, in BALB/c mice, represent ~6% of peripheral blood CD4⁺ T cells (30, 31). We have found that the levels of CD25 expression on CD4⁺ T cells in naïve IFN-γ KO mice are not different from those in WT mice. Similarly, there is no difference in CD25 expression on thymocytes between the two groups (data not shown), which indicates that IFN-γ deficiency is not associated with the lack of thymic production of CD4⁺CD25⁺ regulatory T cells. Accordingly, IFN-γ KO mice do not spontaneously develop autoimmune diseases.

IFN-γ KO mice exhibit enhanced autoimmune response when autoreactive lymphocytes are exogenously stimulated in the presence of a strong adjuvant. The difference in CD25 expression was observed only upon immunization-induced activation of T cells and was pronounced in the peripheral blood 9 days after immunization, a time point that corresponds to the peak activation of peripheral lymphocytes in EAM. Significant differences in percentages of CD4⁺CD25⁺ T cells in the heart were observed only during the acute phase of EAM but not before immunization or during the recovery phase. Our in vitro experiments demonstrated that upon activation, CD4⁺ T cells from IFN-γ KO mice up-regulated CD25 to a significantly smaller degree than those from the WT mice. Thus, the difference in CD25 expression cannot be explained by reduced thymic production of classic regulatory T cells in IFN-γ KO mice but, rather, by the impaired up-regulation of CD25 upon activation.
It has been reported that the CD4⁺CD25⁺ regulatory T cells are characterized by the high expression of CD62L, whereas CD4⁺CD25⁺ activated T cells have low expression of CD62L (32, 33). We found that the predominant majority of CD4⁺CD25⁺ T cells in EAM are CD62Llow (data not shown), further supporting the notion that these T cells represent an activated population.

Our findings suggest that IFN-γ deficiency is associated with the disturbed kinetics of up-regulation of CD25 during CD4⁺ T cell activation. The abnormalities of T cell activation in the absence of IFN-γ are associated with reduced susceptibility to activation-induced cell death, which is critical for physiologic termination of T cell responses (34). We demonstrate that CD4⁺ T cells from IFN-γ KO mice are less susceptible to apoptosis, which accords with observations in other animal models (35). IL-2 receptor signaling has been shown to be involved in inducing not only prosurvival but also proapoptotic pathways and is critical for activation-induced cell death in CD4⁺ T cells (36). CD25 expression leads to increased affinity of IL-2 receptor and, therefore, might contribute to the shift from prosurvival to proapoptotic signaling (36). In this regard, we found that CD4⁺CD25⁺ T cells are more apoptosis-prone than CD4⁺CD25⁻ T cells. The kinetics as well as the degree of activation-induced expression of CD25 may determine to which extent the effector CD4⁺ T cells expand or contract. Thus, the impaired up-regulation of CD25 on CD4⁺ T cells in IFN-γ deficiency may be linked to the inability to physiologically terminate the expansion of the effector T cells and, therefore, to promote autoimmune responses. The importance of such dysregulation in autoimmune myocardiitis is manifested by the resulting expansion of the autoaggressive CD4⁺CD25⁺ T cells.

We have also found increased expression of CD44 on T cells in IFN-γ KO mice both in the periphery and the heart, indicative of a higher proportion of antigen-experienced population (37). Similarly, Chu et al. (22) demonstrated the expansion of CD44highCD4⁺ T cells in IFN-γ KO in a model of experimental autoimmune encephalomyelitis. The disproportionate expansion of CD44high T cells is likely the result of impaired activation-induced cell death in the absence of IFN-γ, which targets activated, but not naïve, T cells.

Myocardial infiltration by the aggressive CD4⁺ T cells in IFN-γ KO mice results in increased cardiac damage, remodeling, dysfunction, and eventually heart failure. Cardiac damage due to inflammation leads to replacement fibrosis, which is associated with the development of DCM in the KO mice. The latter observation agrees with studies in humans demonstrating that hearts from DCM patients have increased collagen content (38).

These findings provide insights into the immune mechanisms leading to the expansion of autoaggressive T cell populations in the context of IFN-γ deficiency. Further studies are needed to delineate the exact mechanisms by which the enhanced inflammation in the myocardium causes cardiomyocyte damage and dysfunction leading to heart failure. Our findings not only may be relevant to the immune involvement in the development of cardiomyopathy but also may be applicable to a broad range of autoimmune diseases.

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