Neutralization of Endogenous Tumor Necrosis Factor Ameliorates the Severity of Myosin-Induced Myocarditis

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Tumor necrosis factor (TNF) and interferon gamma (IFN-γ) are pleuripotent cytokines and have multiple functions during the inflammatory response. Using a murine model of autoimmune myocarditis, we studied the role of TNF and IFN-γ in myocardial inflammation. Neutralizing monoclonal antibodies against TNF-α/β and IFN-γ were administered to myosin-immunized A/J mice to assess the effect on the severity of myocardial inflammation. Anti-TNF treatment significantly reduced the severity of myocarditis compared with rat immunoglobulin G or saline controls (p<0.0007) when given before myosin immunization. Myosin-specific lymph node T-cell proliferation studies showed no difference in the proliferative response between the anti-TNF–treated mice and controls. Administration of anti-TNF to mice after myosin immunization had no effect on the severity of inflammation. This suggests that TNF is an important mediator early in the pathogenesis of myocardial inflammation in this model of myocarditis. Neutralization of IFN-γ significantly increased the severity of myocarditis compared with rat immunoglobulin G and saline controls (p<0.0065), suggesting that IFN-γ may function as an important regulatory cytokine early in the pathogenesis of myocardial inflammation. Understanding the functions of cytokines during the inflammatory response to myocardial injury may provide important information on possible methods to limit myocardial damage. (Circulation Research 1992;70:856–863)

KEY WORDS • myocarditis • tumor necrosis factor • interferon gamma • cytokines

Injury to the myocardium can result from a variety of causes, including ischemia, allograft rejection, autoimmune diseases, infection, and anthracycline drugs. Regardless of the cause of injury, an inflammatory response results in order to begin repair and restoration of the injured myocardium. Though inflammation is clearly beneficial in mediating myocardial tissue repair, there can be pathological consequences of the inflammatory response. For example, in models of ischemic injury to the myocardium, both activation of the complement cascade1-4 and enhanced neutrophil transendothelial migration5-8 are involved in augmenting tissue injury induced by ischemia. Weisman et al9 demonstrated that administration of soluble human complement receptor type 1 to rats with experimental myocardial infarction reduced the severity of inflammation and necrosis. Several studies10-12 suggest that blockade of leukocyte adhesion is important in limiting inflammatory injury. Cytokines may also have an important effect on the myocardium. As shown by the studies of Gulick et al,13 both tumor necrosis factor (TNF) and interleukin-1 can inhibit the response of isolated myocytes to β-adrenergic stimuli. These findings could have important implications concerning the role of inflammatory cytokines in accentuating impaired myocardial performance during an inflammatory response to cardiac injury. In addition, Lane et al14 recently reported that administration of lipopolysaccharide to Coxsackievirus B3–resistant B10.A mice rendered them susceptible to Coxsackievirus B3–induced myocarditis. Many cytokines such as interleukin-1, TNF, and interferon gamma (IFN-γ) are produced in response to lipopolysaccharide administration, and this study suggests that inflammatory cytokines may play an important role in determining susceptibility to viral myocarditis. It is clear that identification of the pathways by which viable myocardium can be damaged during the inflammatory response to different causes of injury will be important in finding ways to limit additional tissue damage incurred and to preserve cardiac function during the process of tissue repair.

We were interested to learn more about the role of cytokines in the induction and progression of myocardial injury that results specifically from the inflammatory response, distinct from other contributing causes of injury. In a murine model of autoimmune myocarditis, originally described by Neu et al15 and shown by us to be mediated by T lymphocytes,16 cardiac-specific inflammation is induced by immunizing susceptible mouse strains with purified mouse cardiac myosin emulsified in complete Freund’s adjuvant (CFA). This model system is ideal for studying the inflammatory response in the heart, because the use of inbred mice eliminates genetic
variation in the inflammatory response, inflammation is reproducingly induced within a well-defined time course of 21 days, and the entire myocardium can be examined histologically, thereby reducing the sampling error that can complicate studies on myocarditis.\textsuperscript{17} We studied the roles of TNF and IFN-\(\gamma\) in this disease because of their importance in both the inductive and effector phases of the immune response.\textsuperscript{18,19} In this report we show that TNF is an important cytokine in mediating myocardial damage in this model of cardiac-specific inflammation and that IFN-\(\gamma\) is an important regulatory cytokine in the pathogenesis of cardiac inflammation in this disease.

Materials and Methods

Mice

Male A/J mice (The Jackson Laboratory, Bar Harbor, Me.) were housed in microisolator cages (Allentown Caging, Allentown, Pa.) and fed autoclaved chow and water. All animals were 2–4 months of age at the time of experiment.

Immunizations

Mouse cardiac myosin was isolated and purifed as previously described.\textsuperscript{16} One hundred micrograms of purified myosin was emulsified in CFA (Difco, Detroit, Mich.) and injected subcutaneously on day 1 and day 7. Five hundred nanograms of pertussis toxin (List Biological Laboratories, Inc., Campbell, Calif.) was injected intraperitoneally on day 1 only. The mice were killed on day 21, and their hearts were removed for histological analysis.

Antibodies

TN3-19.12 was a generous gift from Dr. Robert Schreiber, University of Chicago, St. Louis, Mo. This Armenian hamster immunoglobulin G (IgG) monoclonal antibody (mAb) neutralizes both murine TNF-\(\alpha\) and TNF-\(\beta\) (lymphotoxin).\textsuperscript{20} Therefore, in this report, we will use the term TNF to refer to the activities of both TNF-\(\alpha\) and TNF-\(\beta\). H22, also a gift from Dr. Robert Schreiber, is a neutralizing Armenian hamster IgG mAb against murine IFN-\(\gamma\).\textsuperscript{20} Both TN3-19.12 and H22 contain <5 endotoxin units/ml when analyzed by the Limulus polyphemus amebocyte assay. Rat IgG was obtained from Sigma Chemical Co., St. Louis, Mo. Syrian hamster IgG was obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa. Because neither purified Armenian hamster IgG nor an irrelevant control Armenian hamster mAb are currently available, there was no optimal control antibody for these experiments. It is important to note that these experiments are internally controlled, because both TN3-19.12 and H22 are mAbs derived from Armenian hamsters; thus, effects due to administration of the mAb itself would be equally apparent in both mAb treatment groups. Nonetheless, we wanted to show that xenogenic immune responses did not affect the results of these studies; therefore, we used two different kinds of rodent IgG, compared them with saline, and found that there were no differences in the severity of myocarditis induced (Tables 1 and 2). Each antibody was diluted in sterile saline, and 250 \(\mu\)g was administered intraperitoneally every 7 days. This dose of mAb was shown to provide adequate neutralization of endogenously produced cytokine, and the dosing schedule was chosen because the half-life of hamster mAb in mice is \(\sim 7–14\) days.\textsuperscript{20,21} For the saline controls, an equal volume of sterile saline was injected intraperitoneally every 7 days.

Lymph Node T-cell Proliferation Studies

One hundred micrograms of cardiac myosin emulsified in CFA was prepared as described above and injected subcutaneously into male A/J mice. The mice also received 500 ng pertussis toxin by intraperitoneal injection at the time of myosin immunization. Seven days after the immunization, the draining lymph nodes were aseptically removed into Hanks’ balanced salt solution (GIBCO, Grand Island, N.Y.) that contained 1% newborn calf serum and gentamicin. A single cell suspension was obtained, and the cells were washed three times and resuspended in RPMI (GIBCO) containing 0.5% normal A/J serum, 1 mM HEPEPS, 1 mM pyruvate, 1 mM nonessential amino acids (GIBCO), 2 mM glutamine, 50 \(\mu\)M 2-mercaptoethanol, and 50 \(\mu\)g/ml gentamicin. The T-cell proliferative response to cardiac myosin was determined by culturing 5 x 10\(^5\) lymph node cells in 96-well flat-bottomed plates (Costar) with media alone, myosin, or sonicated Mycobacterium tuberculosis (H37Ra, Difco), referred to as PPD, as the positive control. After 72 hours at 37°C, 0.4 \(\mu\)Ci [methyl-\(3^H\)]thymidine (New England Nuclear, Boston) was added to each well, and the cultures were harvested 24 hours later. Because native cardiac myosin is toxic to lymph node cells in culture, a cyanogen bromide (CNBr, Sigma) digest of purified cardiac myosin was prepared as follows: Two hundred milligrams of CNBr was added to 15 mg cardiac myosin and 10 ml of 70% formic acid. After 12 hours at room temperature, 70 ml distilled H\(_2\)O was added to stop the reaction, and the mixture was frozen at \(-20^\circ\)C. The formic acid and CNBr were removed by lyophilization. The myosin polypeptides were resuspended in H\(_2\)O, sterilely filtered, and kept at 4°C until use. Peptide concentration was estimated by the Bradford assay (Bio-Rad Laboratories, Richmond, Calif.).

Induction of Myocarditis

The induction of myocarditis and histological methods have been previously described.\textsuperscript{16} Briefly, mouse cardiac myosin was isolated and purified, and 100 \(\mu\)g purified myosin was emulsified in CFA and injected subcutaneously on day 1 and day 7. Five hundred nanograms of pertussis toxin was injected intraperitoneally on day 1 only. Immunization of A/J mice with myosin/CFA and pertussis toxin reproducibly causes myocarditis in \(\sim 96\)% of the mice.\textsuperscript{16} Though the mice showed no specific signs of congestive heart failure, such as respiratory difficulty, peripheral edema, or ascites, some mice had ruffled fur and appeared weak and ill. On day 21, the mice were killed by cervical dislocation; the hearts were excised and immediately placed into 10% buffered formalin. In many cases, necrosis of the epicardium was visibly apparent on excision. The tissue was embedded in paraffin, and serial 8–10-\(\mu\)m sections were made through the entire heart and stained with hematoxylin and eosin. Myocarditis was defined as the presence of a lymphocytic infiltrate and adjacent myocardial necrosis.\textsuperscript{17} The lesions were composed of an
interstitial infiltrate predominantly consisting of macrophages, neutrophils, and T cells. The surrounding myocytes appeared to be injured and in various stages of cell death with vacuolated cytoplasm, fragmented cell membranes, and pyknotic nuclei. The severity of myocarditis was determined by estimating the percentage of inflamed myocardium in the most severely affected cross section after all sections throughout each heart were reviewed according to a previously published grading scale. This grading scale was developed such that by visual inspection of the sections under low power (×20), we could view the entire cross section and reproducibly separate hearts with >50%, 10–50%, <10%, or no inflammation. Because no assumptions about the normal distribution of myocarditis severity in a population of A/J mice were made with these methods, we could then use a nonparametric analysis to test if significant differences were present between the treatment groups. Each heart was assigned points in order to compare the severity of myocarditis between different treatment groups according to the following scale: normal, no inflammation (one point); minimal, 0–10% inflammation (two points); moderate, 10–50% inflammation (three points); severe, >50% inflammation (four points). All estimations were made under low power by one reviewer (S.C.S.) who was blinded to the identity of the slide. Representative examples of the histological findings in these studies are found in Figure 1.

**Statistics**

Differences in the severity of myocarditis between the treatment groups were assessed by the Mann-Whitney rank-sum test using the STATVIEW II program (Abacus Concepts, Inc., Berkeley, Calif.) on a Macintosh IIcx computer.

**Results**

**Neutralization of TNF Ameliorates the Severity of Myocarditis**

The effect of TNF neutralization on the severity of myocarditis was studied by initiating anti-TNF mAb treatment before myosin/CFA immunization. A neutralizing dose (250 μg) of the anti-TNF αβ mAb TN3-19.12 was given on days 0, 6, 13, and 20, and the mice were immunized with myosin/CFA on days 1 and 7. The results of these studies are shown in Table 1, and photomicrographs of the histological findings are shown in Figure 1. Of the 35 A/J mice treated with TN3-19.12, eight were normal, 17 had minimal myocardial inflammation, five had moderate myocardial inflammation, and five had severe inflammation. In contrast, none of the 20 mice treated with control rat IgG had normal hearts: six had minimal inflammation, four had moderate inflammation, and 10 had severe inflammation. There was no significant difference in the severity of myocarditis between the rat IgG and saline control groups (p < 0.7). The reduction in the severity of myocardial inflammation found in the TN3-19.12-treated group compared with the rat IgG control group was statistically significant at p < 0.0007. Thus, treatment with anti-TNF mAb dramatically reduced the severity of myocarditis. These results suggest that TNF is an important mediator of myocardial inflammation in this autoimmune disease.

**Anti–IFN-γ Increases the Severity of Myocarditis**

IFN-γ has important effects on many aspects of the immune response, including activation of macrophages, induction of major histocompatibility complex class I and II gene expression, and regulation of proliferation of both T and B lymphocytes. In addition, many of these effects are modulated by TNF. Therefore, we wanted to study how neutralization of IFN-γ affected the severity of myocardial inflammation. A neutralizing dose (250 μg) of the anti–IFN-γ mAb H22 was given on days 0, 6, 13, and 20, and the mice were immunized with myosin/CFA on days 1 and 7. Unexpectedly, we found that the severity of myocarditis was significantly increased. The results of these studies are shown in Table 1, and photomicrographs of the histological findings are shown in Figure 1. Of the 23 mice treated with H22, 21 had severe myocarditis, one had minimal inflammation, and one had a normal myocardium. The severity of myocarditis found in the H22-treated group was significantly different from both the rat IgG control group (p < 0.0065) and the TN3-19.12–treated group (p < 0.0001). This suggests that IFN-γ may have an important role in modulating the immune response during the pathogenesis of myocarditis in this model.

**Neutralization of TNF or IFN-γ Does Not Affect the Proliferation of Lymph Node T Cells to Cardiac Myosin**

Neutralization of TNF or IFN-γ had opposite effects on the severity of myocarditis as determined by histological analysis. If TNF or IFN-γ were critical to T-cell activation, then the effect of neutralization of these cytokines might be reflected in the lymph node T-cell response to cardiac myosin. Using a standard in vitro assay system that measures antigen-specific proliferation of primed lymph node T cells, we measured the proliferative response of lymph node T cells from myosin-immunized A/J mice treated with either TN3-19.12, H22, rat IgG, hamster IgG, or saline to CNBr-digested cardiac myosin. CNBr-digested myosin was used for these lymph node proliferation studies because native myosin is toxic to lymph node T cells in vitro (authors’ unpublished observations). As shown in Figure 2, lymph node T cells from mice treated with rat IgG, hamster IgG, or saline proliferated to CNBr-digested myosin in a dose-dependent manner and showed a vigorous response to the positive control antigen PPD. The proliferation of lymph node T cells from mice treated with TN3-19.12 (anti-TNF) or H22 (anti–IFN-γ) to CNBr-digested myosin was not different compared with the control treatments (Figure 2). These results show that neutralization of the endogenously produced TNF or IFN-γ in response to immunization with cardiac myosin/CFA does not affect the activation of myosin-reactive T cells. Therefore, the reduction in myocardial inflammation seen with anti-TNF and the augmentation of inflammation seen with anti–IFN-γ most likely does not occur at the level of T-cell activation.

**TNF Is Important Early in the Pathogenesis of Myocarditis**

Neutralization of endogenously produced TNF significantly reduced the severity of myocarditis when the anti-TNF mAb TN3-19.12 was administered before immunization, as shown in Table 1. We were interested...
to learn if the severity of myocarditis could be reduced when anti-TNF treatment was initiated after immunization with cardiac myosin. To further explore this question, we first immunized A/J mice with cardiac myosin/CFA according to the usual protocol on days 1 and 7 and then killed them by cervical dislocation every 4 days until day 20 in order to determine when histological evidence of inflammation first appears. As shown in Table 2, myocarditis can be seen as early as day 8, but it is most prevalent between days 16 and 20. We then administered either TN3-19.12 or hamster IgG according to two different schedules as shown in Table 3, beginning on day 6, before histological evidence of inflammation, or on day 10, after inflammation is first apparent. In these studies, TN3-19.12 administration after the initial immunization did not reduce the severity of myocarditis compared with hamster IgG, regardless of whether anti-TNF treatment was started on day

FIGURE 1. Photomicrographs of hematoxylin and eosin-stained cross sections showing the effects of anti-tumor necrosis factor (TN3-19.12) and anti-interferon gamma (H22) monoclonal antibody treatment on the severity of myocarditis in myosin-immunized A/J mice (magnification, ×304). Panel A: Minimal myocarditis from a TN3-19.12–treated mouse. Panel B: Severe myocarditis from an H22–treated mouse. Panel C: Moderate myocarditis from a rat IgG treated mouse.
TABLE 1. Effect of Anticytokine Antibodies on the Severity of Myocarditis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Myocarditis severity</th>
<th>Incidence of disease (%)</th>
<th>Average severity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Minimal</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>TN3-19.12</td>
<td>8</td>
<td>17</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>H22 (anti-IFN-γ)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Rat IgG</td>
<td>0</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Values are the numbers of mice in each treatment group according to the severity of myocarditis as determined by histological analysis and described in “Materials and Methods.” TNF, tumor necrosis factor; IFN-γ, interferon gamma; IgG, immunoglobulin G. Severity of myocarditis is ranked as follows: normal, 1; minimal, 2; moderate, 3; and severe, 4. TN3-19.12, H22, rat IgG, or saline was given by intraperitoneal injection the day before myosin immunization and every 7 days thereafter until the end of the experiment on day 21. The results are pooled from four separate experiments. Differences in the severity of myocarditis between the groups were determined by the Mann-Whitney rank-sum test.22

*TN3-19.12 vs. rat IgG; †H22 vs. rat IgG; ‡H22 vs. TN3-19.12; §rat IgG vs. saline.

6 or 10. This is in contrast to the results shown in Table 1, where TN3-19.12 markedly reduced the severity of myocarditis when given before the initial myosin/CFA immunization. From these studies we conclude that TNF is an important mediator during the inductive phase of myosin-induced autoimmune myocarditis but that neutralization of TNF from days 6 to 21 after the first immunization did not affect the ongoing inflammatory process in this disease model.

Discussion

The studies presented here used cytokine-specific mAb in a model of cardiac-specific inflammation to provide insight into the roles of TNF and IFN-γ during inflammatory processes in the heart. We have demonstrated that TNF is a critical cytokine early in the inflammatory process and that IFN-γ has unexpected anti-inflammatory functions in this model of cardiac-specific inflammation.

These data clearly show that neutralization of endogenous TNF ameliorates the severity of myosin-induced autoimmune myocarditis. Because TN3-19.12 recognizes TNF-α and TNF-β, the decrease in severity of myocarditis seen could be due to neutralization of either or both of these cytokines. When TN3-19.12 was administered before immunization with myosin/CFA, the severity of myocardial inflammation was significantly reduced. In addition, anti-TNF treatment did not block the activation or proliferation of cardiac myosin-reactive T cells. Possible key effects of TNF on the process of myocardial inflammation include its role in major histocompatibility complex class I and class II gene expression and intercellular adhesion molecule expression. Because TNF is secreted by activated macrophages and can act synergistically with IFN-γ to increase the expression of both major histocompatibility complex class I24-27 and class II28-32 molecules, interference with these events could have important effects on the processing and presentation of cardiac myosin peptides to T cells. The lymph node proliferation studies showed that the T-cell response to cardiac myosin was preserved, regardless of whether the mice received neutralizing doses of anti-TNF or anti-IFN-γ mAb. This suggests that, at least in the regional lymph nodes of these mice, processing and presentation of cardiac myosin peptides was sufficient to activate T

FIGURE 2. Graph showing the effect of anti–tumor necrosis factor (TN3-19.12) and anti–interferon gamma (H22) treatment on lymph node T-cell proliferation to cyanogen bromide (CNBr)–digested myosin in myosin-immunized A/J mice. Controls (hamster IgG [HIG], rat IgG [RIG], and saline) are indicated by open symbols. Values represent the incorporation of [3H]thymidine in counts per minute (cpm) in triplicate cultures (after subtraction of background cpm) and are representative of three separate experiments. Standard deviations were <5%. The responses to the positive control antigen PPD are as follows: TN3-19.12, 32,716 cpm; H22, 31,260 cpm; HIG, 50,411 cpm; RIG, 34,815 cpm; and saline, 54,802 cpm.

TABLE 2. Time Course for the Induction of Autoimmune Myocarditis

<table>
<thead>
<tr>
<th>Days after immunization</th>
<th>Normal Severity</th>
<th>Myocarditis severity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Minimal</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Values are the numbers of mice evaluated after death by cervical dislocation at each day of study according to the histological severity of myocarditis as described in “Materials and Methods.” Immunizations of myosin emulsified in complete Freund’s adjuvant were given on days 1 and 7, as per the usual treatment protocol.
TABLE 3. Anti-Tumor Necrosis Factor Treatment Does Not Reduce the Severity of Myocarditis After Initial Myosin Immunization

<table>
<thead>
<tr>
<th>Antibody regimen</th>
<th>Myocarditis severity</th>
<th>Disease incidence (%)</th>
<th>Average severity p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Minimal</td>
<td>Moderate</td>
</tr>
<tr>
<td>Days 6, 13, and 20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamster IgG</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>TN3-19.12 (anti-TNF)</td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Days 10 and 17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamster IgG</td>
<td>1</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>TN3-19.12 (anti-TNF)</td>
<td>1</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are the numbers of mice in each treatment group according to the severity of myocarditis as determined by histological analysis and described in “Materials and Methods.” IgG; immunoglobulin G; TNF; tumor necrosis factor; NS, not significant. Severity of myocarditis is ranked as follows: normal, 1; minimal, 2; moderate 3, severe, 4. Immunizations of myosin emulsified in complete Freund’s adjuvant were given on days 1 and 7, according to the usual treatment protocol. TN3-19.12 or control hamster IgG were given by intraperitoneal injection on the days shown. The results are pooled from two separate experiments. Differences in the severity of myocarditis between the groups were determined by the Mann-Whitney rank-sum test.

...cells, but this assay does not test whether these activated T cells reach the myocardium and remain activated. With regard to cellular trafficking, it is well established that TNF-α is important in recruiting immune effector cells to sites of inflammation by increasing the expression of intercellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 on endothelial cells, and thereby enhancing the ability of cells expressing leukocyte function-related antigen-1 to bind to the endothelia and migrate into sites of tissue inflammation. We favor the hypothesis that neutralization of TNF could directly impair the ability of T cells, monocytes, and neutrophils to migrate from the circulation into the myocardium and thus prevent localization of activated immune effector cells. This observation may find important clinical utility in the treatment of cardiac allograft rejection; e.g., transplant recipients could be pretreated with anti-TNF to interfere with several possible steps in the immune reaction against allograft tissue.

The results presented here are similar to the findings of Ruddle et al., who demonstrated that administration of the same anti-TNF mAb (TN3-19.12) to SJL/J mice reduced the severity of experimental allergic encephalomyelitis (EAE) induced by transfer of a myelin basic protein-specific T-cell clone. EAE is a murine model of autoimmune inflammation in the central nervous system, analogous to multiple sclerosis in humans, where immune-mediated demyelination is induced by immunization with purified myelin basic protein emulsified in CFA. In those studies, the mice that developed EAE despite treatment with TN3-19.12 developed less severe disease than those mice that received control antibody, similar to our findings presented in Table 1. In contrast, Teuscher et al. used a polyclonal antibody against TNF-α to treat mice immunized with myelin basic protein and found no reduction in the severity of EAE.

It is possible that differences in the efficacy of TNF neutralization between TN3-19.12 and the polyclonal antisera used by Teuscher et al could explain why no effect on the severity of EAE was seen in their studies. It is also interesting to compare our findings with the effects of TNF-α on lupus nephritis in (NZB×NZW)F1 mice and on diabetes mellitus in nonobese diabetic mice. In both of those mouse strains, direct administration of the cytokine TNF-α attenuated the expression of inflammation. It is important to note that in both of these mouse strains, the onset of autoimmune disease is spontaneous, whereas myosin-induced myocarditis and EAE require induction, either by antigen or by transfer of antigen-reactive T-cell clones. This may be important in understanding the pathogenic mechanisms involved and delivering effective treatment for these different immune-mediated tissue injuries.

The significantly increased severity of inflammation observed in myosin/CFA–immunized mice treated with anti–IFN-γ was surprising and suggests that IFN-γ may play an important role in regulating the inflammatory response to cardiac proteins. The major functions of IFN-γ include activation of macrophages and monocytes and induction of major histocompatibility complex class I and II gene expression. In addition, IFN-γ has been shown to increase expression of ICAM-1 and to inhibit the growth of CD4+ Th2 cells. It is unlikely that systemic neutralization of IFN-γ resulted in decreased antigen-presenting cell function or major histocompatibility complex class I and II gene expression, as this would most likely impair antigen presentation and activation of myosin-reactive T cells. Furthermore, immunohistochemical studies on hearts from mice treated with anti–IFN-γ mAb show abundant major histocompatibility complex class II protein expression (authors’ unpublished observations). It is also unlikely that neutralization of IFN-γ inhibited expression of ICAM-1, since this would act to prevent rather than enhance the immune response. Though the function of CD4+ Th1 and Th2 cells in myosin-induced myocarditis is not known, it is possible that neutralization of IFN-γ causes a predominantly Th2 response to cardiac myosin that ultimately results in greater myocardial destruction. Resistance or susceptibility to Leishmania major infection is a well-studied example of how preferential induction of IFN-γ–producing Th1 cells or interleukin-4–producing Th2 cells directly correlates with resistance or susceptibility to leishmaniasis (reviewed in Reference 42). These studies do not address how IFN-γ acts to modulate the inflammatory response to cardiac myosin, but it is clear that the isolated functions of this cytokine identified by in vitro studies are made considerably more complex when studied in vivo systems.
Interestingly, similar findings were reported by Billiau et al., who demonstrated increased severity of EAE in C57BL/6J mice treated with a neutralizing mAb against IFN-γ. They postulated that the local effects of IFN-γ promote inflammation, whereas the systemic effects are anti-inflammatory, and therefore systemic administration of IFN-γ mAb would neutralize circulating IFN-γ and preferentially promote an enhanced local inflammatory response. This raises an interesting question about where a cytokine mediator is functioning during an inflammatory response and how to effectively deliver a neutralizing antibody to the target tissue. Another possible explanation for our results is that the IFN-γ mAb used in our experiments was immunogenic itself, causing an enhancement of the immune response simply by its antigenic properties. This is unlikely, given the dramatically different effects that anti-TNF or anti-IFN-γ administration had on the severity of myocarditis. Because both the TNF mAb TN3-19.12 and the IFN-γ mAb H22 used in these studies are derived from Armenian hamsters and are endotoxin free, we would expect similar effects on the severity of myocarditis if a xenogenic anti-IgG response were occurring. Furthermore, the T-cell proliferative responses to myosin in the TN3-19.12–treated and H22–treated animals were similar and did not significantly differ from the saline-treated or control antibody–treated groups (Figure 2). These data suggest that the increased severity of myocarditis seen in the H22–treated mice does not result from an enhanced immune response to the antibody molecule.

Anti-TNF treatment that was started before the initial myosin/CFA immunization caused a profound reduction in the severity of myocarditis. These data together with the finding that anti-TNF administration after the initial immunization did not affect the severity of myocarditis suggest that TNF is important early in the processes that lead to myocardial inflammation but may not be critical for the progression of myocardial inflammation once initiated. Other cytokines may antagonize the effects of TNF at later stages in the inflammatory process, or TNF may not be produced later in the course of the disease such that neutralization of TNF has a demonstrable effect on the severity of myocarditis. Furthermore, it is possible that the concentrations of anti-TNF activity present, either locally or systemically, after immunization were insufficient to block progression of inflammation. However, it is also possible that this observation reflects a fundamental aspect of autoimmune processes, in that once the mechanisms of activation and T-cell localization to self-proteins are adequately initiated, the process cannot be modulated by neutralization of a single cytokine, and that control of the immune response may require blocking the function of multiple simultaneously operating pathways.

We believe that TNF may be important at several steps in the initiation of myocardial inflammation in this model and that the effects of TNF neutralization reflect the multiple functions of this cytokine in the inflammatory response, many of which are accentuated or diminished by other cytokines such as IFN-γ. IFN-γ may have an important regulatory function in the pathogenesis of autoimmune myocarditis. Understanding more about the complex interactions of IFN-γ with TNF and other cytokines may provide greater insight into how an individual cytokine functions in the many reactions of an ongoing immune response within the myocardium.

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