Presence of IFN-γ Does Not Indicate Its Necessity for Induction of Coronary Arteritis in an Animal Model of Kawasaki Disease

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*J Immunol* 2004; 173:3492-3503; doi: 10.4049/jimmunol.173.5.3492

http://www.jimmunol.org/content/173/5/3492
Presence of IFN-γ Does Not Indicate Its Necessity for Induction of Coronary Arteritis in an Animal Model of Kawasaki Disease

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Kawasaki disease (KD) is the most common cause of vasculitis affecting children, and is characterized by prolonged fever, nonpurulent conjunctivitis, oral mucosal inflammation, cervical lymphadenopathy, induration and erythema of the hands and feet, and a diffuse polymorphous skin rash (1–3). Although the inflammatory response is found in medium and small vessels throughout the body, the most common site of end organ damage is the coronary arteries. KD is now recognized as the leading cause of acquired heart disease in children in the developed world (4). Despite appropriate therapy with i.v. gammaglobulin, up to 20% of affected children continue to develop coronary artery lesions, when adjusted for body surface area (5). Many gaps still exist in our knowledge of the etiology and pathogenesis of KD, making improvements in therapy difficult.

Although still debated, current evidence suggests that the cause of KD is of an infectious origin, with superantigenic activity leading to massive stimulation of the immune system, resulting in vesSEL wall damage and aneurysm formation in the coronary arteries. The immune response in KD is wide ranging, encompassing both aspects of innate and specific immunity. Activation of both B and T cells has been detected (6–9) together with increased proinflammatory cytokine production, including TNF-α, IL-1, and IL-6 (10). Progression from systemic activation to local inflammation at the vessel wall is evidenced by endothelial cell activation and upregulation of adhesion molecules (11, 12). Presently, the mechanisms leading from immune activation to localized coronary artery damage are not known. In the last decade, considerable evidence has accumulated to suggest that T lymphocytes play a significant role in the pathogenesis of KD. Several studies have demonstrated the presence of infiltrating T cells in the coronary arteries, specifically in coronary artery lesions (13, 14). Upon activation, T cells produce cytokines with multiple functions. IFN-γ is a pleiotropic cytokine predominantly produced by T and NK cells (15). IFN-γ, usually in synergy with TNF-α, takes on important roles in the stimulation of Ag presentation through MHC I and II molecules, activation of macrophages and other immune cells, orchestration of leukocyte-endothelial interactions, control of endothelial cell and vascular smooth muscle cell (SMC) proliferation, and apoptosis (15–19). Several studies have demonstrated an antiproliferative function of IFN-γ on Ag-specific, as well as mitogen-stimulated T cell proliferation (20–24), but its role in vivo inflammatory responses has been more controversial. IFN-γ appears to play a proinflammatory role in autoimmune neuritis (25, 26) and experimental autoimmune myasthenia gravis (27), but an anti-inflammatory function in other autoimmune disease models such as type II collagen-induced arthritis (28–31), experimental autoimmune uveoretinitis (EAU) (32), and experimental allergic encephalomyelitis (EAE) (33, 34). Studies have reported that experimental autoimmune thyroiditis, EAU, and EAE develop in mice lacking IFN-γ (24, 35–37). Additionally, administration of rIFN-γ appears to inhibit the development of diabetes in the NOD mice (38).

References

IFN-γ has also been implicated in coronary vascular diseases, potentiating atherosclerosis and coronary lesions in ApoE-deficient mice, as well as contributing to the pathogenesis of accelerated coronary vascular disease in transplanted hearts (39, 40). Studies addressing the role of IFN-γ in coronary disease secondary to KD have yielded conflicting results. Some have reported high serum levels of both IFN-γ and TNF-α during the acute phase of KD (41, 42), while others demonstrated a decrease in IFN-γ-producing CD4+ T cells (43). All KD studies to date have focused on serum levels of IFN-γ, and no information is available regarding IFN-γ in affected vessels.

Studying affected coronary arteries from children is not possible when even repeated blood sampling is ethically difficult; thus, an animal model is required. We have characterized and refined an animal model of KD, which uses *Lactobacillus casei* cell wall extract (LCWE) to induce coronary arteritis in young mice (44, 45). We have found that LCWE possesses superantigenic activity (45). Immunologic responses to LCWE possess all the hallmarks of a superantigen (SAg)-mediated response: marked proliferation of naïve T cells, nonclassical MHC restriction with a hierarchy in the efficiency of different class II molecules to present this SAg, a requirement for Ag presentation but not processing, and stimulation of T cells in a nonclonal, TCR Vβ-dependent fashion. More importantly, the superantigenic activity is directly correlated with the ability to induce coronary arteritis in mice (45). Humans display marked sensitivity to bacterial SAgS compared with mice, in part due to increased affinity of human MHC class II molecules for bacterial SAgS (46). For this reason, we have refined the model of LCWE-induced coronary arteritis by using genetically modified mice expressing human CD4 and human MHC class II DPQ6 transgenes in a background lacking endogenous murine CD4 (mCD4) and CD8 (mCD8). These DPQ6 transgenic mice effectively circumvent the species barrier, and are phenotypically and functionally normal with respect to Th cell functions (47). They are unique in that they display human-like responsiveness to bacterial SAgS, and are supersensitive to their pathogenic effects (48), including immune response to LCWE and induction of coronary artery disease (45).

In the present study, we examined the role of IFN-γ in the immune response leading to vascular damage in the LCWE-induced coronary arteritis model of KD. We report the presence of IFN-γ in the hearts of affected animals. The kinetics of its production is biphasic, and coincides with the earliest signs of inflammation at the coronary artery, and subsequently coronary artery wall disruption. Interestingly, responses to LCWE were not only intact, but exaggerated in IFN-γ-deficient mice, with participation by the same SAg-responsive TCR Vβ families as in wild-type mice. This translated into a similar incidence of coronary arteritis in both wild-type and IFN-γ-deficient animals. IFN-γ appears to participate in and regulate the immune response to LCWE superantigenic stimulation, but is not absolutely required for the induction of coronary arteritis.

Further understanding of the mechanisms regulating immune activation and localized inflammation in the affected vessels will influence treatment and long-term morbidity and mortality in children with KD.

**Materials and Methods**

**Mice**

Wild-type C57BL/6 mice and IFN-γ-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DPQ6 transgenic mice have been described previously (47). Briefly, mice deficient of either CD4 (mCD4−/−) or CD8α (mCD8−/−) were generated by targeted gene disruption and homologous recombination in embryonic stem cells (49, 50). They were then bred together to generate the mCD4 mCD8− double knockout (51). Mice expressing the human CD4 transgene were bred into the mCD4 mCD8− double knockout mouse (52). Concurrently, the human MHC class II DPQ6 transgenic mice with co-injected DPQ6α and β genomic DNA fragments under control of their endogenous promoters (53) were bred into the mCD4 mCD8− background. These two strains of mutant mice were then bred together to obtain human CD4 DPQ6 mCD8− (DPQ6 transgenic), which were backcrossed eight generations into C57BL/6 mice.

**Preparation of LCWE**

LCWE was prepared, as previously described (44). Briefly, *L. casei* bacteria (American Type Culture Collection, Manassas, VA) (ATCC 11578) were first cultured in Lactobacillus MR5 broth (Difco, Detroit, MI) at 37°C (on a shaker platform), and then harvested by centrifugation (10,000 × g, 40 min) and growth of supernatant culture after extensive washes in PBS (pH 7.2), bacteria were lysed by overnight incubation (at room temperature on a shaker platform) with 4% SDS (EM Science, Gibbstown, NJ) (in 10 vol), followed by 10 washes with PBS. Sequential incubations with 250 μg/ml RNase, DNaseI, and trypsin (Sigma-Aldrich, St. Louis, MO) were performed to remove any adherent material from the cell walls. Each incubation was for 4 h at 37°C at twice the packing volume, and was followed by 2 washes in PBS or 4 washes after the incubation with trypsin. The pellet was then sonicated (5 g of packed wet weight in 15 ml of PBS) in a continuous dry ice/ethanol bath for 2 h at a pulse setting of 5.0 (10-s pulse/5-s pause) (500 Sonic Dismembrator with a one-half-inch tapped horn and tapered microtip; one-eighth-inch diameter, tuned to vibrate at a fixed frequency of 20 Khz; Fisher Scientific, Nepean, Canada). Following 1-h centrifugation at 40,000 × g, the supernatant containing the cell wall extract was harvested for injection. The concentration of the preparation was based on a phenol-sulfuric acid colorimetric determination of the rhamnose content and expressed in mg/ml final concentration in PBS (54).

**RNA extraction**

Four- to 5-wk-old mice were injected i.p. with PBS alone or PBS containing 1 mg of LCWE or 10 μg of staphylococcal enterotoxin B (SEB) (Toxin Technology, Sarasota, FL). At various time points postinjection, mice were sacrificed, and their hearts and spleens were removed and immediately homogenized in TRizol reagent (Invitrogen Life Technologies, Palo Alto, CA) using a Polytron PT 1200 homogenizer (KINEMATICA, Lucerne, Switzerland). Total RNA was isolated as per protocol (Invitrogen Life Technologies). Briefly, heart and spleen homogenates were first centrifuged (10,000 × g for 10 min), and the supernatant was collected. After two chloroform (Sigma-Aldrich) treatments, the RNA was precipitated with isopropanol (Sigma-Aldrich). RNA pellets were then washed with 70% ethanol, air-dried, and dissolved in sterile water containing 0.1% diethylpyrocarbonate (Sigma-Aldrich). RNA concentration was determined by OD measured with a spectrophotometer (Fisher Scientific).

**RT-PCR**

cDNA was synthesized using murine leukemia virus reverse transcriptase and the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). cDNA was amplified by PCR in a GeneAmp 9700 thermal cycler (Applied Biosystems) as per protocol. Briefly, each 50 μl amplification mixture contained 2 μl of cDNA, 0.2 μM dNTPs (Applied Biosystems), 1 U of *Taq* polymerase, 5 μl of 10× reaction buffer (Brinkmann Instruments, Hamburg, Germany), and 0.2–1.0 μM each of 5′ and 3′ primers, and sterile water. To ensure that there were no false positive results, a reaction mixture containing no cDNA was also included in every PCR run. After an initial step at 94°C for 2.5 min to activate the polymerase, each cycle consisted of 15 s of denaturation at 94°C, 45 s of annealing at appropriate temperatures indicated for each primer pair in Table I, followed by 60-s extension at 72°C. At the end of the last cycle, there was an additional 5-min extension step at 72°C. Thirty to 34 cycles were performed for all primer pairs. PCR products were resolved by electrophoresis in a 1% agarose gel stained with ethidium bromide. Relative intensity of the PCR products was quantified using the Kodak Digital Science camera and Kodak 1D2.02 software (Eastman Kodak, Rochester, NY). Amplified products were expressed as a ratio to the housekeeping gene product hypoxanthine phosphoribosyltransferase (HPRT). Preliminary experiments were performed to ensure that the number of PCR cycles for each primer pair was within the exponential phase of the amplification curve. This allowed semiquantitative comparisons to be made between the levels of expression of the various mRNA species in different samples. In all cases, primers were mRNA specific in that the recognition sites of the 5′ and 3′ primers resided in separate exons in the genomic sequence, i.e., intron spanning, thus amplifying only sequences from mRNA. Sequences, annealing temperature, and predicted PCR product sizes for each primer pair are shown in Table I. For each
experiment, the ratios of IFN-γ, TNF-α, or IL-1β to HPRT net intensity from all mice at a given time point were averaged together, and the SE was calculated.

Flow cytometric detection of intracellular IFN-γ

Four- to 5-wk-old mice were injected i.p. with 500 μl of PBS alone or PBS containing 1 mg of LCWE. At various time points postinjection, splenocytes were prepared into single cell suspension, and permeabilized at 2 × 10^6 cells/ml permeabilizing solution (PBS containing 20 mM EDTA, 2% FBS, and 0.1% saponin (Sigma-Aldrich)). After 10- to 20-min incubation at room temperature, cells were centrifuged and supernatant was removed. They were then stained in 100 μl of permeabilizing solution containing the appropriate dilution of PE-conjugated rat anti-mouse IFN-γ Ab (clone XM1G2, BD Biosciences, Mississauga, Canada). Control samples were stained similarly with an isotype-matched Ab (BD Biosciences). Samples were analyzed using CelleQuest software on a FACSscan or FACSComp (BD Biosciences).

In collective experiments, splenocytes from IFN-γ-deficient or wild-type C57BL/6 mice were cultured either in complete medium alone (RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 μM 2-ME, and 10 mM HEPES), or in complete medium containing various concentrations of LCWE. All cultures were set up in triplicates in 96-well plates (Nunc), incubated at 37°C in 5% CO2 for 72 h. They were pulsed with 1 μCi/well [3H]thymidine (Amersham Biosciences, Baie d’Urfe, Canada) for the last 6 h of incubation.

Statistical analysis

Where applicable, two-way ANOVA test and Fisher’s exact test were used for statistical analysis. A p value of ≤0.05 was considered significant.

Results

Rapid IFN-γ mRNA expression following LCWE stimulation

We previously established that the induction of coronary arteritis in mice correlates directly with the presence of superantigenic activity in LCWE. This ability to induce localized disease in the coronary arteries is specific to LCWE, as other bacterial SAgs including SEB and toxic shock syndrome toxin-1 (TSST-1) do not cause coronary arteritis (45). To investigate whether the production of IFN-γ in response to LCWE may account for disease induction capabilities, we examined the kinetics of IFN-γ mRNA expression in splenocytes from DQ6 transgenic mice following injection with LCWE, SEB, or PBS alone. RT-PCR was performed to determine levels of IFN-γ mRNA expression in relation to that of the housekeeping gene HPRT. Fig. 1A illustrates the relative amounts of IFN-γ and HPRT mRNA as reflected by the intensity of the 688- and 1114-bp PCR products, respectively. As expected, samples of cDNA at all time points and all experimental conditions showed comparable expression of HPRT. In contrast, IFN-γ mRNA was not constitutively expressed. There was rapid production of IFN-γ mRNA peaking within 12 h post-LCWE injection, followed by a steady decline in message production until 24–72 h, when levels were barely detectable (Fig. 1A). This early response is readily seen when production of IFN-γ mRNA is normalized to the housekeeping gene, HPRT (Fig. 1B). Similarly, SEB elicited a rapid response with high levels of IFN-γ mRNA at 12 h postinjection, which started to decline by 18 h, and took 7–14 days to return to baseline (Fig. 1B). No IFN-γ mRNA was detected in splenocytes from mice injected with PBS at any of the same time points (Fig. 1B).

Translation of IFN-γ mRNA message into protein

To determine whether IFN-γ protein was produced in response to LCWE, we examined the intracellular expression of IFN-γ protein in splenocytes by both flow cytometry and confocal microscopy.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
<th>Primer Sequence (5’-3’)</th>
<th>Annealing Temperature (°C)</th>
<th>PCR Product Size (bp)</th>
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<tr>
<td>IFN-γ</td>
<td>GAA AGC CTA GAA AGT CTT GAT AAT AAC T</td>
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<td>688</td>
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<tr>
<td>TNF-α</td>
<td>ATG AGC ACA GAG AGC ATG ACG GCC</td>
<td>60</td>
<td>200</td>
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<td></td>
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<tr>
<td>IL-1β</td>
<td>TGA CGG ACC CCA AAA GAT GAA</td>
<td>58</td>
<td>468</td>
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<tr>
<td>HPRT</td>
<td>GCC TTC CTC CTC AGA CGG CTT T</td>
<td>60</td>
<td>1114</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. RT-PCR primer pairs: sequence, annealing temperature, and expected PCR product size
No intracellular IFN-\(\gamma\) protein was detected in splenocytes at any time point post-PBS injection, but expression of IFN-\(\gamma\) protein was rapidly induced following LCWE injection (Fig. 2A). By 12 h post-LCWE injection, intracellular IFN-\(\gamma\) protein was detected in 8% of total splenocytes. The percentage of IFN-\(\gamma\)-expressing cells dropped to 4% by 18 h, and to undetectable levels by 24 h. Similarly, when cryosections of spleen from mice injected with LCWE were prepared and examined for intracellular expression of IFN-\(\gamma\) by confocal microscopy, cells stained positive for IFN-\(\gamma\) were detected in follicles as early as 6–12 h post-LCWE injection (Fig. 2B). No signal was observed in samples from PBS-injected mice, or those stained with an isotype-matched Ab (Fig. 2B and data not shown). The appearance of IFN-\(\gamma\)-producing cells within 12 h post-LCWE injection was consistent with results obtained by flow cytometry and RT-PCR, confirming that the LCWE-induced IFN-\(\gamma\) message was rapidly translated into protein.

**Bimodal expression of IFN-\(\gamma\) mRNA in affected cardiac tissue**

The activation of the systemic immune response in KD leads to inflammation at the coronary arteries. To investigate the kinetics of IFN-\(\gamma\) expression in the affected vasculature during evolution of the disease, we performed RT-PCR on mRNA obtained from mice injected with LCWE and sacrificed from 18 h to 60 days postinjection. As seen in Fig. 3A, IFN-\(\gamma\) message was markedly increased in samples from mice sacrificed at 3 and 28 days postinjection. Relatively less IFN-\(\gamma\) was detected at other time points, although still much more than that found in PBS-injected mice, which was mainly undetectable. Figures represent single experiments, using mice matched for age and LCWE preparation to minimize possible confounding factors. Fig. 3B represents the relative levels of IFN-\(\gamma\) mRNA in 3–5 animals at each time point after...
local production of IFN-γ in affected cardiac tissue. These observations indicate that the histopathological changes seen in the coronary arteries following LCWE, but not SEB injection, are reflected in differences in local production of IFN-γ in affected tissue.

**Localization of IFN-γ protein to the affected vessel wall**

To identify the regions of the heart populated by IFN-γ-producing cells post-LCWE injection, confocal microscopy was used to visualize intracellular IFN-γ using fluorescence-tagged mAbs. Fig. 3C shows IFN-γ staining of the base of the heart containing the aorta and coronary artery as captured by serial cryosections prepared from an animal 42 days post-LCWE injection. IFN-γ was detected in cells located in the outer regions of the coronary vessel wall corresponding to the adventitia where inflammatory cells are found on H&E staining. Staining with an isotype-matched Ab showed no signal. Our data demonstrate that local production of IFN-γ in affected vessels begins at the level of transcription as evidenced by mRNA production, and is followed by translation into IFN-γ protein. The site of IFN-γ production correlates with the site of the localized immune cell infiltrate in the affected vessel wall.

**Persistent inflammatory infiltrate in affected cardiac tissue**

To determine the kinetics of the localized inflammatory response in the coronary arteries during disease evolution, coronary arteries were identified, isolated, and preserved in cryosections from mice following LCWE injection. The first indication of inflammation in the heart could be detected as a mononuclear cell infiltrate in the adventitia of coronary arteries of LCWE-injected mice as early as days 3–7 postinjection (Fig. 4). The inflammatory process began in the adventitia and was accentuated in the microvasculature, especially the capillaries. This is identical with the early histopathological changes seen in human KD, with initiation of the inflammatory process in the adventitia with involvement of the vasovasorum (55–59). Coronary inflammation continued, with maximum cellular infiltrates at day 28 post-LCWE injection (Fig. 4). Similarly, inflammation persisted well into the disease and continued to be readily detected at day 42 (Fig. 4). This is associated with elastin breakdown and vessel wall disruption visualized by EVG staining (Fig. 4F) on the day 42 serial section corresponding to the H&E-stained section in Fig. 4E. Even at 6 mo post-LCWE injection, there is evidence of mild inflammation and microvascular changes, again in accord with human data describing chronic, long-term histological changes in children up to 23 years postacute KD (60). Cardiac tissue from mice injected with PBS did not exhibit inflammation at any time during the course of the study. Similarly, no cellular infiltrates or coronary artery inflammation was observed in the coronary arteries of mice following SEB injection.

**Marked proliferation of IFN-γ-deficient lymphocytes in response to LCWE**

To investigate the contribution of IFN-γ to the immune response following LCWE stimulation, we examined the in vitro proliferative response to LCWE in IFN-γ-deficient mice. Splenocytes from IFN-γ-deficient mice and wild-type controls were cultured with different concentrations of LCWE, and their proliferative responses were measured after 3 days. As previously reported (45), LCWE induced marked proliferation of naïve T cells from C57BL/6 mice in a dose-dependent fashion with maximal response at 50 μg/ml. Interestingly, splenocytes from IFN-γ-deficient mice proliferated more vigorously than splenocytes from wild-type mice at all LCWE concentrations tested (Fig. 5A). Whereas proliferation of IFN-γ-deficient splenocytes was detected at LCWE concentrations as low as 0.8 μg/ml, that of the wild-type splenocytes was detected only at 12.5 μg/ml. Furthermore, while proliferative response of the wild-type cells dropped beyond an LCWE dose of 50 μg/ml, that of splenocytes from IFN-γ-deficient animals was still...
Cultured with either LCWE or anti-CD3/H9280 SAg-reactive T cells, C57BL/6 splenocytes were cultured with either H9253, which splenocytes from IFN-H9252 SAg in LCWE. To determine whether IFN-H9252 LCWE, T cells expressing TCR V-H9253 families were 8, 14, and 13, respectively, in C57BL/6 mice were used as controls. As described above, C57BL/6 wild-type mice developed maximal in situ inflammation at the coronary arteries 28 days after LCWE injection (Fig. 6). Interestingly, inflammatory coronary vessel disease was also found in IFN-H9252-deficient animals. The pathology of the coronary artery lesions at 28 days post-LCWE injection was comparable in the wild-type and IFN-H9252-deficient mice, which in turn, appears to regulate the immune response to LCWE.

**FIGURE 4.** Persistent inflammatory infiltrate in affected cardiac tissue. Cryosections of cardiac tissue from mice injected with either PBS or LCWE were stained with H&E or EVG stain, and examined for the presence of coronary arteritis. Cardiac tissue was harvested on day 28 post-PBS injection (A), or days 3 (B), 7 (C), 28 (D), and 42 (E) post-LCWE injection. F, A serial section corresponding to the H&E-stained section in E was stained with EVG stain. All images were collected with a ×20 objective.

Expansion of the same SAg-reactive TCR Vβ families in IFN-γ-deficient and wild-type mice in response to LCWE

The observation that IFN-γ-deficient splenocytes responded to LCWE more vigorously than those of wild-type mice raised the question as to whether the same subpopulations of T cells are expanded in response to LCWE in these animals (45). To determine the TCR Vβ profile of the J relaxations were 2, 4, and 6 skewing was observed in vivo in animals in which splenocytes from IFN-γ-deficient and wild-type mice were cultured with either LCWE or anti-CD3 plus anti-CD28 Abs, and assayed 3 days later (data not shown).

Production of IFN-γ by LCWE-reactive T cells

We have shown that, following LCWE stimulation, IFN-γ production is up-regulated at both the mRNA and protein levels, and that IFN-γ participates in and regulates the immune response to the SAg in LCWE. To determine whether IFN-γ is produced by the SAg-reactive T cells, C57BL/6 splenocytes were cultured with either medium only or LCWE for 14–16 h. Cells were harvested, and stained for TCR Vβ2, 4, and 6. Positive cells were sorted and analyzed for intracellular IFN-γ by flow cytometry. As seen in Fig. 5C, whereas only 2–4% of total splenocytes expressed intracellular IFN-γ in the unsorted population, IFN-γ was detected in 79% of the TCR Vβ2-, 4-, and 6-expressing T cell subpopulation.

Taken together, our findings demonstrate that upon stimulation by LCWE, similar TCR Vβ families are expanded in the wild-type and IFN-γ-deficient mice, and that these SAg-activated T cells produce IFN-γ, which in turn, appears to regulate the immune response to LCWE.

Similar incidence of LCWE-induced coronary arteritis in IFN-γ-deficient and wild-type mice

Our observation that IFN-γ regulates the immune response to LCWE, and that IFN-γ expression in the heart was coincident with the induction of local vascular inflammation raised the question as to whether IFN-γ was necessary for induction of disease. To address this question, we injected IFN-γ-deficient mice with either LCWE or PBS alone, and assayed for the presence of coronary artery inflammation. IFN-γ-deficient mice are on the C57BL/6 background, having been backcrossed >20 generations; thus, wild-type C57BL/6 mice were used as controls. As described above, C57BL/6 wild-type mice developed maximal inflammation at the coronary arteries 28 days after LCWE injection (Fig. 6). Interestingly, inflammatory coronary vessel disease was also found in IFN-γ-deficient animals. The pathology of the coronary artery lesions at 28 days post-LCWE injection was comparable in the wild-type and IFN-γ-deficient mice (Fig. 6). H&E staining of cardiac tissue obtained from affected IFN-γ-deficient or wild-type mice revealed similar populations of inflammatory cells, mainly consisting of small mononuclear cells with a high nuclear to cytoplasmic ratio. Immunohistochemical analysis showed these to be CD3+ lymphocytes in both groups of mice (our unpublished data). Furthermore, the incidence of mice developing inflammation in the
cardiac tissues following LCWE injection was similar in both the IFN-γ-deficient and wild-type mice (Table II). In both strains, the incidence of coronary inflammation varied between 80 and 100%, depending on the preparation of LCWE used. None of the animals injected with PBS alone in either mouse strain showed any signs of inflammation. Taken together, these observations suggest that IFN-γ is not required for the induction of coronary arteritis.

Similar disease course in IFN-γ-deficient and wild-type mice

To examine whether IFN-γ influences the kinetics of disease evolution, cryosections of coronary arteries from either C57BL/6 or IFN-γ-deficient mice injected with LCWE and sacrificed at various time points were stained with H&E. Interestingly, IFN-γ-deficient mice develop coronary arteritis with similar disease course as in the wild-type animals (Fig. 7). The histopathological features appeared similar and followed the same kinetics in both murine strains. In both the wild-type and IFN-γ knockout animals, an immune cellular infiltrate was detected at the coronary arteries of affected mice as early as day 7 post-LCWE injection. The inflammatory infiltrate that was comprised of small mononuclear cells with a high nuclear to cytoplasmic ratio was detected in the adventitia of the coronary arteries, and migrated into the rest of vessel and the surrounding myocardium as the disease progressed. The inflammation of the coronary arteries was still readily detected 42 days after LCWE injection in both the wild-type and IFN-γ-deficient hearts. No cellular infiltrate was detected in cardiac tissues prepared from PBS-injected mice of either strain (data not shown).

Similar cytokine profile in the IFN-γ-deficient and wild-type mice

The finding of coronary disease in the absence of IFN-γ prompted the question as to whether other inflammatory cytokines were up-regulated to compensate for the lack of endogenous IFN-γ. To investigate this possibility, we examined mRNA levels of two key inflammatory mediators, TNF-α and IL-1β, in IFN-γ-deficient and
FIGURE 6. Comparable incidence of LCWE-induced coronary arteritis in IFN-γ-deficient and wild-type mice. Cryosections of hearts harvested 28 days postinjection from C57BL/6 mice (A) and IFN-γ-deficient mice (B) were stained with H&E. Both images were collected with a ×20 objective.

wild-type mice following LCWE injection. TNF-α cDNA was amplified from cardiac tissue of IFN-γ knockout or wild-type mice sacrificed between 3 and 42 days post-LCWE injection, important time points previously identified. Fig. 8A represents the relative levels of TNF-α mRNA in 4–11 animals at each time point after normalization to the housekeeping gene HPRT. As seen, TNF-α message was present in the heart of wild-type mice by day 3 post-LCWE injection, and persisted through day 42. Similarly, TNF-α mRNA expression in the heart of IFN-γ-deficient mice was detectable at all time points examined. More importantly, the relative levels and kinetics of TNF-α mRNA production in the heart of IFN-γ-deficient and wild-type animals were the same during the development of LCWE-induced coronary arteritis. No statistically significant difference was detected in the TNF-α message levels between the IFN-γ-deficient and wild-type mice at any time point.

Similar to TNF-α, IL-1β mRNA was detected by day 3 post-LCWE injection, and persisted to day 42. Fig. 8B shows the relative IL-1β mRNA levels in the heart of IFN-γ-deficient and wild-type mice at the specified time points, as described above. The relative levels were the same in the wild-type and IFN-γ-deficient mice at all time points. The absence of IFN-γ, the signature Th1 cytokine, may also lead to a change in production of Th2 cytokines. We examined the production of IL-4 and IL-10, cytokines important in the Th2 immune response, and found undetectable levels of both cytokines in both the IFN-γ-deficient and wild-type mice from days 3–42 post-LCWE injection (data not shown). Taken together, these data demonstrate that the same soluble mediators of inflammation are involved in the induction of LCWE-induced coronary arteritis in IFN-γ-deficient and in wild-type mice.

Discussion

KD is a clinical syndrome characterized by the classic signs of inflammation: redness, heat, and swelling in affected parts of the body. The etiology appears to be of infectious origin, leading to massive stimulation of the immune system, resulting in vascular damage and aneurysm formation in the coronary arteries. The road from multisystem inflammation to localized damage in the coronary arteries is not known, and the nature of the localized inflammatory response and mechanisms involved in development of coronary artery lesions are not clearly understood. Cardiac tissue is not readily available from children with KD, thus necessitating a disease model. LCWE-induced coronary arteritis in mice is an accurate model of KD, sharing identical time course, pathology, response to treatment, and susceptible populations to the human disease (44, 45, 61, 62), and provides the opportunity to examine the immune response at the site of vascular damage.

T lymphocytes play an important role in the pathogenesis of human KD. T cells, localized to the site of aneurysm formation, have been found in the coronary arteries of affected children (13, 14). Consistent with the hypothesis advocating a SAg etiology, skewing of the TCR Vβ repertoire to SAg-reactive Vβ families has been found in some outbreaks of KD (63). Studies concentrating on TSST-1 have found selective expansion of TCR Vβ2* and TCR Vβ8.1* families of reactive T cells during the acute phase of KD (64, 65). Autopsy evidence supporting the role of these T cells in local inflammation in the affected myocardium and coronary arteries has also been documented, with selective expansion of TCR Vβ2+ families in the affected tissue. Sequence analysis of these TCR Vβ2+ T cells showed extensive junctional region diversity, consistent with a nonclonal proliferation characteristic of SAg stimulation (66). These observations support the concept that the activation of T cells is involved in cardiovascular damage associated with KD. T cell activation is characterized by production of IFN-γ. Studies addressing the role of IFN-γ in the pathogenesis of KD have yielded conflicting results. In some studies, elevated serum levels of IFN-γ were detected (41, 42). Others reported a decrease in the number of IFN-γ-producing CD3+ cells in the periphery (43). These studies have focused on peripheral blood levels of IFN-γ, and no data are available concerning the affected coronary arteries. In this study, we examined the production of IFN-γ at the site of end organ damage in a murine model of KD, and report the biphasic expression of IFN-γ in the affected vessels during the development of coronary artery disease. Despite the prominent role of IFN-γ in the peripheral immune response to LCWE stimulation, ablation of IFN-γ led to an exaggerated proliferative response, and did not affect the incidence of coronary arteritis. Thus, IFN-γ appears to participate in and regulate the immune response during development of coronary arteritis, but is not necessary for the induction of coronary disease.

The immune response to LCWE SAg stimulation occurred rapidly, resulting in maximal IFN-γ mRNA production in the peripheral lymphoid tissue within 12 h following injection. The levels

Table II. Incidence of LCWE-induced coronary arteritis in IFN-γ-deficient and wild-type mice

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Incidence of Coronary Arteritis</th>
<th>LCWE</th>
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</thead>
<tbody>
<tr>
<td>IFN-γ+/+</td>
<td>0/4</td>
<td>10/11</td>
</tr>
<tr>
<td>IFN-γ−/−</td>
<td>0/4</td>
<td>15/15</td>
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*Four- to 5-wk-old IFN-γ+/+ or IFN-γ−/− mice were injected i.p. with PBS or PBS containing 1 mg of LCWE. Cryosection of cardiac tissues was prepared 28 days postinjection, stained with H&E, and examined for the presence of cellular infiltrate at the coronary.

*Incidence of coronary arteritis is presented as the number of mice showing inflammatory infiltrates at the coronary over the total of mice injected. No statistical significance was detected between the incidence of disease in the IFN-γ-deficient and wild-type animals (p = 0.4).
Movement of the immune response from the peripheral lymphoid tissue to the site of end organ damage is supported by both histological findings and the kinetics of IFN-γ production. Following LCWE injection, infiltrating cells were detected in the coronary arteries of affected mice as early as day 3–7 postinjection (Fig. 4). First the adventitia, and then the medium and the rest of the vessel wall were invaded by inflammatory cells, with maximal cellular infiltrate observed at day 28 postinjection. Inflammatory cells could be seen migrating from the microvasculature supplying the affected tissue. The chronicity is similar to human KD (60), with cellular infiltrate observed well into the disease at 6 mo post-LCWE injection (our unpublished data). The striking similarity of the histopathology and kinetics of disease between LCWE-induced coronary arteritis in mice and human KD further validates the accuracy of the animal model. Interestingly, the detection of IFN-γ mRNA coincided precisely with presence of cellular infiltrates. Although the immune cellular infiltrate was present in the heart of affected mice as early as day 3, and persisted at least until day 42 post-LCWE injection, the production of IFN-γ was more specific, with an initial peak coinciding with the first appearance of cellular infiltrates, and a later peak at ~days 28–42, coinciding with aneurysm formation. The nonrandom production of IFN-γ suggests a role for this cytokine in the very important stages of disease development.

Among coronary vessel diseases, aneurysm formation is unique to KD. We have found that coronary artery lesions continue to occur in up to 20% of appropriately treated patients when adjusted for body surface area in affected children (69). This is consistent with data found in other centers (5). Untreated, the incidence of coronary artery lesions is as high as 70% when corrected for body surface area. Coronary artery lesions are most commonly detected in the subacute stage of KD. The subacute stage is characterized clinically by resolution of the fever and the classic systemic symptoms associated with KD, plus desquamation of the skin at the fingers and toes. During this phase, which lasts from ~day 10 to 6 wk after onset of fever, there is most likely ongoing inflammation, with continued elevation of the platelet count and erythrocyte sedimentation rate. In the mouse model, vascular damage, remodeling, and aneurysm formation start to occur during the subacute stage, at days 28–42 post-LCWE injection. Interestingly, the second peak of IFN-γ mRNA production in affected cardiac tissue declined promptly, and reached basal levels by 24–72 h postinjection (Fig. 1). The increase in IFN-γ message levels induced by LCWE was accompanied by IFN-γ protein production as detected by intracellular flow cytometry and confocal microscopy (Fig. 2). The kinetics of IFN-γ mRNA production following LCWE is typical for a bacterial SAg (45, 67). The differential levels of expression of IFN-γ induced by LCWE and SEB observed may simply reflect the potency of each agent, and hence, the strength of the immune response elicited.

Three to 7 days following LCWE injection, a peak in IFN-γ mRNA production was found in affected hearts. This coincides with the time of first appearance of inflammatory infiltrate in the coronary vessel wall (Fig. 4) (44, 45). As observed in the spleen, IFN-γ mRNA in the heart was translated into protein. The IFN-γ is localized to the adventitia of the vessel wall, where mononuclear cells are predominant around the microvasculature (Fig. 3). IFN-γ is produced primarily by the Th1 subset of reactive lymphocytes, as well as NK cells (15). Immunohistochemical determinations have found that T cells, but not NK cells, are present within the inflammatory infiltrate in the coronary artery wall (our unpublished data). Together, these suggest a role for a Th1 cell-mediated local immune response in target tissue. Consistent with this notion, we have found low or undetectable mRNA levels of Th2 cytokines such as IL-4 and IL-10 in the same affected heart tissue (our unpublished data). Induction of coronary arteritis is specific to LCWE SAg activity, but not other SAgs such as TSST-1 or SEB (Fig. 3). The systemic immune response elicited by LCWE is typical of bacterial SAgs. However, the localized immune response appears to be distinct and specific to the coronary disease-inducing ability of LCWE. Whereas SEB induced a robust peripheral immune response with similar kinetics as that induced by LCWE, no vascular disease and no inflammatory infiltrate were observed in the hearts of these SEB-injected mice. This would argue against bystander activation of autoreactive T cells by any SAg as a mechanism for progression of disease to the target vessels. Superantigenic activity in LCWE may induce massive activation of the peripheral lymphocyte population, including a subpopulation of autoreactive T cells that target the coronary vessels, promoting inflammation and, ultimately, damage of vascular tissue. This is consistent with the current belief that KD belongs to the spectrum between an infectious disease and true autoimmune disease (68).
vascular damage in LCWE-induced coronary arteritis. The generation and modulation of the immune response leading to organ following LCWE injection support a role for this cytokine in IL-1β/H9253 f ni respectively. Two-way ANOVA was used for statistical analysis. No significance of elastin fragmentation and vessel wall disruption is evident in up to 50% of affected mice at this time point (Fig. 4). Elastin breakdown is the hallmark of aneurysm formation and an important event in the evolution of vascular damage leading to thinning and weakening of the vessel wall and aneurysm formation (70). In addition to elastin breakdown in the extracellular matrix, the cellular components of the vessel wall may also contribute to aneurysm formation. Several studies have reported an inhibitory role of IFN-γ on the proliferation of vascular smooth muscle cells (16, 17). Furthermore, IFN-γ has been shown to induce apoptosis of SMC (18). IFN-γ and TNF-α work synergistically to enhance apoptosis of SMC via the Fas-Fas ligand pathway (19). Imbalance in SMC growth regulation due to IFN-γ-induced apoptosis and inhibition of vascular SMC proliferation, together with degradation of the extracellular matrix scaffolding characterized by elastin breakdown may be the important steps leading to development of coronary artery lesions. Taken together, our findings on the kinetics of IFN-γ production in the periphery, as well as in the target organ following LCWE injection support a role for this cytokine in the generation and modulation of the immune response leading to vascular damage in LCWE-induced coronary arteritis.

The role of IFN-γ in in vivo models of inflammatory disease has been controversial. A proinflammatory role for this cytokine has been reported for certain autoimmune disease models such as autoimmune neuritis (25, 26) or experimental autoimmune myasthenia gravis (27), but IFN-γ appears to be anti-inflammatory in other models such as EAU (32), EAE (33, 34), type II collagen-induced arthritis (28–31), and, more recently, diabetes (38) and autoimmune myocarditis (71, 72). Our observation that lymphocytes from IFN-γ-deficient mice proliferated more vigorously to LCWE than those from wild-type animals demonstrates an important regulatory function for IFN-γ, which, in this case, is antiproliferative. This is consistent with reports that IFN-γ inhibited proliferation of T lymphocytes in response to both specific Ags and mitogens (20–24). IFN-γ has been demonstrated to exert its antiproliferative effect through the induction of NO and reactive oxygen intermediates by macrophages (20, 21). The ability of IFN-γ to mediate SAg-induced T cell tolerance through its action on Gr-1+ myeloid cells has also been reported (73). Furthermore, IFN-γ has also been demonstrated to act directly on the T cells to inhibit their proliferation in response to specific Ag (22). The intact, but exaggerated periphery immune responses mediated by LCWE in IFN-γ knockout mice may contribute to the development of coronary arteritis in IFN-γ-deficient animals. We have determined that LCWE possesses superantigenic activity that results in the activation of T cell subpopulations expressing CDR Vβ2, 4, and 6 in both wild-type and IFN-γ-deficient mice (45). Staining of the T cell repertoire with selective expansion of SAg-reactive TCR Vβ2, 4, and 6 families was observed both in vivo and in vitro. Thus, higher proliferation of IFN-γ-deficient splenocytes reflects an antiproliferative function of IFN-γ, allowing expansion of SAg-reactive TCR Vβ families. This is further evidenced by the increased expansion of these LCWE-responsive TCR Vβ2, 4, and 6 families in the IFN-γ-deficient mice in comparison with wild-type mice. Our finding that the same TCR Vβ family expressing T cells was stimulated by LCWE SAg in wild-type and IFN-γ-deficient animals is consistent with the hypothesis that a specific subpopulation of T cells mediates this disease and that IFN-γ knockout mice develop the same disease as the wild-type mice. Interestingly, LCWE-induced IFN-γ production is specific to T cells expressing SAg-reactive TCR Vβ2, 4, and 6. Staining of these cells revealed that 79% were positive for intracellular IFN-γ. This suggests that in wild-type mice, IFN-γ produced by the LCWE-responsive T cells may trigger a negative feedback on their expansion. Regulation of immune responses or inflammation by negative feedback, either through actions of soluble mediators or of intracellular negative regulator factors such as the suppressors of cytokine signaling proteins, has been well established (73–75). A recent study by Cauley et al. (76) has demonstrated that IFN-γ-mediated SAg-induced T cell suppression is dependent on the action of myeloid cells. Under normal physiological conditions, inflammation is eventually resolved, and inflammatory cells are cleared in a timely manner. In autoimmune disease, including our model, inflammation persists and leads to end organ damage, in our case, coronary artery wall damage.

The incidence of coronary disease is similar between IFN-γ knockout and wild-type mice. Furthermore, both strains of mice exhibited similar histopathological features and disease course. Cytokine gene-targeted mutant mice have been shown to compensate immunologically by increasing the production and effectiveness of other cytokines (77). In some experimental autoimmune disease models, disease develops in the absence of IFN-γ (24, 35–37). In these animal models, target organ damage was caused by compensatory inflammatory mediators and/or effector cell populations in the IFN-γ-deficient mice in comparison with wild-type mice.

FIGURE 8. Similar levels of TNF-α and IL-1β mRNA in cardiac tissue of IFN-γ-deficient and wild-type mice post-LCWE injection. Four- to 5-wk-old mice were injected with LCWE, and heart removed at various time points postinjection. RT-PCR was performed to detect TNF-α or IL-1β and HPRT mRNA levels. The net intensity ratios between A, TNF-α or B, IL-1β and HPRT bands were measured and averaged for all mice at any given time point (n = 5–11 for wild-type mice; n = 4–10 for IFN-γ-deficient mice). □ and ■, Represent wild-type and IFN-γ-deficient mice, respectively. Two-way ANOVA was used for statistical analysis. No significant difference was observed between any group (p < 0.05).
animals. In other disease models, including autoimmune neuritis and autoimmune myasthenia gravis, the disease course is unaffected in the absence of IFN-γ. Similarly, in our model, the disease course is unchanged in the absence of IFN-γ, and the same inflammatory cytokines appear to be involved in the induction of LCWE-induced coronary arthritis in the wild-type and IFN-γ-deficient mice. There was no switch to the Th2 cytokine profile in the knockout mice with undetectable IL-4 and IL-10 mRNA in both the wild-type and IFN-γ-deficient mice at all times during disease evolution. This is consistent with reports studying EAE models (37). An important role for the proinflammatory cytokines TNF-α and IL-1β has been reported for human KD (10, 78–80). Similar mRNA levels of these key proinflammatory cytokines were observed in both the wild-type and IFN-γ-deficient animals throughout disease evolution. The observation that IFN-γ-deficient mice expressed similar TNF-α mRNA levels as the wild-type mice is, thus, consistent with the ability of these mice to develop disease following LCWE injection. Similar to the soluble mediators of the disease, the cellular components of coronary disease are unchanged in the absence of IFN-γ. H&E staining of cardiac tissue obtained from affected IFN-γ-deficient and wild-type mice showed similar populations of inflammatory cells infiltrating the coronary arteries. These cells were identified as T lymphocytes by cell surface staining. This contrasts with some other disease models, including EAE, experimental autoimmune thyroiditis, and EAU, in which the inflammatory cell populations were altered in the absence of IFN-γ (24, 35–37).

In summary, we report the biphasic production of IFN-γ in coronary arteries of mice following LCWE injection. Immune activation was evidenced by rapid production of IFN-γ in the peripheral lymphoid tissue, followed by migration to the site of end organ damage. The coincident expression and colocalization of IFN-γ with the inflammatory infiltrate in affected vessels, and production of IFN-γ by LCWE SAg-reactive T cells support an important role for IFN-γ in development of coronary artery lesions. Ablation of IFN-γ led to an exaggerated peripheral immune response and a similar incidence of coronary disease, demonstrating that IFN-γ participates in and regulates the immune response, but is not necessary for the induction of coronary artery disease. Improved understanding of the mechanisms by which inflammation leads to vascular damage will influence treatment and long-term morbidity and mortality in children with KD.

Acknowledgments

We acknowledge Mark Duvvuri for technical assistance, and Joyce Hui-Yuen for assistance in microscopy.

References
