The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 15 2004

Key Roles of CD4+ T Cells and IFN-γ in the Development of Abdominal Aortic Aneurysms in a Murine Model[1] FREE

Wanfen Xiong; ... et. al

J Immunol (2004) 172 (4): 2607–2612.
https://doi.org/10.4049/jimmunol.172.4.2607

Key Roles of CD4$^+$ T Cells and IFN-γ in the Development of Abdominal Aortic Aneurysms in a Murine Model[1]

Wanfen Xiong,* Yong Zhao,*§ Amy Prall,* Timothy C. Greiner,† and B. Timothy Baxter[2]*‡

Abdominal aortic aneurysm (AAA) is one of a number of diseases associated with a prominent inflammatory cell infiltrate and local destruction of structural matrix macromolecules. This inflammatory infiltrate is predominately composed of T lymphocytes and macrophages. Delineating specific contribution of these inflammatory cells and their cytokines in AAA formation is the key to understanding AAA and other chronic inflammatory disease processes. Our previous studies have demonstrated that macrophages are the major source of matrix metalloproteinase-9, which is required for aneurysmal degeneration in the murine AAA model. However, the role of CD4$^+$ T cells, the most abundant infiltrates in aneurysmal aortic tissue, is uncertain. In the present study, we found that in the absence of CD4$^+$ T cells, mice are resistant to aneurysm induction. Previous studies have shown that IFN-γ levels are increased in AAA. IFN-γ is a main product of T cells. Intraperitoneal IFN-γ was able to partially reconstitute aneurysms in CD4$^{-/-}$ mice. Furthermore, mice with a targeted deletion of IFN-γ have attenuation of MMP expression and inhibition of aneurysm development. Aneurysms in IFN-$\gamma^{-/-}$ mice can be reconstituted by reinfusion of competent splenocytes from the corresponding wild-type mice. This study demonstrates the pivotal role that T cells and the T cell cytokine, IFN-γ, play in orchestrating matrix remodeling in AAA. This study has important implications for other degenerative diseases associated with matrix destruction. ***The Journal of Immunology*****, 2004, 172: 2607–2612.**

Abdominal aortic aneurysms (AAA)[3] represent a common and lethal disorder. Earlier concepts of aneurysm expansion envisaged a simple degenerative process but immunohistochemical, cellular, and molecular biological studies of human tissues and animal models of AAA have consistently shown large numbers of inflammatory cells, elevated levels of cytokines and matrix metalloproteinases (MMPs), and destruction of the elastic media (1, 2). Although many aspects of AAA development are undefined, these observations have led to a paradigm shift in which AAA is seen as a complex remodeling rather than a simple degeneration process. The inflammatory infiltrate, which is temporally and spatially associated with disruption of the orderly lamellar structure of the aortic media, appears to play an etiologic role in AAA development and progression directly through its ability to secrete elevated levels of MMPs (3), or indirectly, by secreting cytokines, including IFN-γ and TNF-α, which induce resident mesenchymal cell MMP productions (4–6). Increased proteolytic activity of MMPs results in weakening of the structural matrix of the abdominal aortic wall. The MMPs that have been implicated directly in AAA are MMP-9, MMP-2, MMP-1, and MMP-12 (7–12).

MMPs are a family of Ca^{2+}-activated, Zn^{2+}-dependent endopeptidases that are able to degrade components of extracellular matrix (ECM) by their concerted actions (13). MMP-9 is one of the most abundant elastolytic proteinases secreted by human AAA tissues. It is primarily produced by aneurysm-infiltrating macrophages at the sites of tissue damage and its expression appears to correlate with increasing aneurysm diameter (14, 15). MMP-2 expression is elevated in human AAA (9, 16, 17). It is primarily produced by resident mesenchymal cells in AAA (9).

Analysis of the inflamed aneurysm wall has revealed the presence of a large number of activated T lymphocytes and macrophages (18, 19), implicating these cells as possible mediators in the disease processes. Monocytes are recruited to sites of tissue injury or chronic inflammation by cell-derived cytokines and chemotactic factors. Recent studies using a murine aneurysm model have revealed that macrophage-derived MMP-9 and smooth muscle cell (SMC)-secreted MMP-2 work in concert to produce aneurysms (8). However, the role of CD4$^+$ T cells, the most abundant infiltrates in aneurysmal aortic tissue, remains elusive. IFN-γ, a major inflammatory product of T cells, is a potent activator of macrophage and a cytokine known to regulate MMPs (20). IFN-γ levels are elevated in AAA (21). Therefore, it is reasonable to speculate that the IFN-γ may stimulate MMP production from macrophages and SMC, orchestrating progressive destruction of the normal orderly lamellar architecture in AAA.

The studies on aortic tissue samples from human aneurysms inevitably deal with a late phase of disease and do not necessarily reflect the conditions that initiated aortic dilatation. A reliable animal model is essential to better understand the mechanisms that both initiate and lead to progression of aortic dilatation. Currently, there are three common methods of aortic aneurysm induction including: transient intraluminal elastase perfusion; periaortic application of $CaCl_2$; and angiotensin II-infusion in aopE-deficient mice. To study the mechanisms of aneurysm formation and development, we have been using and characterized the $CaCl_2$-induced aneurysm murine

Departments of *Surgery, †Pathology and Microbiology, and ‡Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, NE 68198; and §Transplantation Biology Research Division, Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

Received for publication August 28, 2003. Accepted for publication December 2, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by National Institutes of Health Grant 5RO1HL62400-02, National Institutes of Health Cardiovascular Research Training Program 5T32HL07888-05, and American Heart Association Grant 974015N (to B.T.B.).

[2] Address correspondence and reprint requests to Dr. B. Timothy Baxter, Surgery Research Department, Lied Transplant Center Room 9727, 987690 University of Nebraska Medical Center, Omaha, NE 68198-7690. E-mail address: btbaxter@unmc.edu

[3] Abbreviations used in this paper: AAA, abdominal aortic aneurysm; MMP, matrix metalloproteinase; ECM, extracellular matrix; SMC, smooth muscle cell; WT, wild type; rmIFN-γ, recombinant murine IFN-γ; hIFN-γ, human IFN-γ.

0022-1767/04/$02.00

model, which emphasizes the role of the inflammatory infiltrate and metalloproteinases in the aneurysmal degeneration process that is remarkably similar to that found in human AAA (8).

In an attempt to understand the inflammatory reaction and subsequent matrix degradation mediated by $CD4^+$ T lymphocytes, we have investigated the role of the $CD4^+$ T lymphocytes in AAA pathogenesis using the murine model. These studies demonstrated that CD4-deficient mice were resistant to aneurysm formation. Because IFN-γ levels are increased in human AAA and IFN-γ is a product of T cells, we went on to define the specific role of IFN-γ. We found that deficiency of IFN-γ in mice prevent aneurysm formation. Importantly, aneurysms can be largely reconstituted in the $CD4^{-/-}$ mice by IFN-γ injection or infusion of competent splenocytes into IFN-$\gamma^{-/-}$ mice. $CD4^{-/-}$ mice and IFN-$\gamma^{-/-}$ mice demonstrate decreased MMP production in the aorta. Conversely, in vitro treatment of macrophages and SMC by IFN-γ increases MMP-9 and MMP-2 production, respectively. The $CD4^+$ T cells and its product, IFN-γ, are important factors in the pathogenesis of AAA by virtue of their ability to induce MMP expression. Thus, these studies delineate the key role invading T lymphocytes play in regulation of the extracellular matrix.

Materials and Methods

Reagents

Recombinant murine IFN-γ (rmIFN-γ) and human IFN-γ (hIFN-γ) were purchased from BD PharMingen (San Diego, CA). Tissue culture medium and FBS were purchased from Life Technologies (Grand Island, NY). The collagen preparation used was Vitrogen 100 (Cohesion Technology, Palo Alto, CA), which is ~95% type I collagen and ~5% type III collagen.

Mice

The homozygous CD4 gene knockout ($CD4^{-/-}$) mice and IFN-γ gene knockout (IFN-$\gamma^{-/-}$) mice bred on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). Normal C57BL/6 mice (The Jackson Laboratory) were used as controls for $CD4^{-/-}$ and IFN-$\gamma^{-/-}$ mice. Both male and female knockout mice were used in a random fashion.

Aneurysm induction model

Mice, at age 8 wk, underwent surgery as described previously (8). Briefly, the mice were anesthetized and underwent laparotomy. The abdominal aorta between the renal arteries and bifurcation of the iliac arteries was isolated from the surrounding retroperitoneal structures. The diameter of the aorta was measured in triplicate midway between the renal artery origin and iliac artery bifurcation. After baseline measurements, 0.25 M $CaCl_2$ was applied to the external surface of the aorta for 15 min. The aorta was rinsed with 0.9% sterile saline and the incision was closed. NaCl (0.9%) was substituted for $CaCl_2$ in sham control mice. Eight weeks later, the mice underwent laparotomy and dissection. Measurements were repeated at the same location in the mid-infrarenal aorta. Typically, there was diffuse, homogeneous dilatation of the infrarenal aorta. The aorta was collected for zymographic analysis of MMP proteins. For histological studies, the aorta was perfusion-fixed with 10% neutral buffered formalin.

Histology and microscopy

Masson's trichrome staining: After perfusion-fixation with 10% neutral buffered formalin, abdominal aortic tissues were embedded in paraffin and cut into 4-μm sections. The slides were stained with hematoxylin, crocein scarlet, acid fuchsin, and aniline blue (Sigma-Aldrich, St. Louis, MO). Each staining cycle alternated between fixing and washing procedures. The slides were examined and photographed using light microscopy (Kodak, Tokyo, Japan) (×20).

Immunohistochemistry

Mice underwent AAA induction according to the method described above. Four mice in each group were sacrificed at 8 wk for T cell staining performed on paraffin-embedded 4-μm aortic sections. The sections were incubated with a polyclonal rabbit anti-CD3α Ab (BD PharMingen) and diluted 1/20 for 30 min at 37°C. The sections were then briefly washed in citrate solution and subsequently incubated with the secondary Ab which is a biotin-conjugated goat anti-rabbit IgG. T cell staining was examined using light microscopy (×100). Positive controls and nonimmune negative controls were performed. CD3-positive cells were graded in the aortic media and adventitia by a pathologist unaware of the genotype or treatment. Four separate sections from each aorta were stained and evaluated, and the mean grade was reported.

IFN-γ treatment

The day after NaCl or $CaCl_2$ treatment, groups of eight $CD4^{-/-}$ mice were injected i.p. with 50,000 U of rmIFN-γ, twice weekly for a period of 3 wk. For injection control, after $CaCl_2$ aneurysm induction, $CD4^{-/-}$ mice ($n = 5$) were injected i.p. with the same volume of vehicle, PBS. Eight weeks later, the mice underwent a second laparotomy and the aortas were exposed and measured before being removed for zymography and histology.

Isolation and infusion of splenocytes

C57BL/6 and $CD4^{-/-}$ mice were sacrificed. Spleens were aseptically removed and teased apart between two sterile slides. Cells were isolated and resuspended in 1 ml RBC lysis buffer (Tris and NH_4CL, pH 7.2). After 1 min, cells were washed with RPMI 1640 medium twice and pelleted to remove cellular debris. Cells were plated in RPMI 1640 supplemented with 10% heat inactivated FBS and incubated at 37°C, 5% CO_2 for 1 h. The suspending cells were collected, pelleted, and resuspended in PBS. Groups of IFN-$\gamma^{-/-}$ mice were injected with 5×10^7 splenocytes from wild-type (WT) or CD4-deficient mice via the tail vein 1 day before AAA induction. A second splenocyte infusion was repeated a week later to booster cell number.

Isolation and culture of mouse peritoneal macrophages

Mouse macrophages were isolated from peritoneal fluid. C57BL/6 mice were sacrificed. Peritoneal macrophages were collected, washed, and resuspended in RPMI 1640 medium. Cells were then plated at 1×10^5 cells/well on 12-well plates, and plates coated with fibrillar collagen (1.5 mg/ml, prepared as suggested by the manufacturer; 0.15 ml/cm^2). Cells were incubated in collagen-coated plates containing RPMI 1640 with 10% FBS medium at 37°C for 2 h, followed by rinses to remove nonadherent cells. The cells then were incubated in RPMI 1640 and treated with or without rmIFN-γ at a concentration of 0.1 and 1.0 ng/ml for 24 h. The conditioned medium was collected and spun at $3000 \times g$ at 4°C for 30 min to remove cell debris. The effects of IFN-γ on MMP production were compared with untreated cells. The specific activity of mrIFN-γ was $>2 \times 10^7$ U/mg.

Isolation and culture of human SMC

Infrarenal aortic tissues were obtained at organ procurement for transplantation. Informed consent was obtained for tissue collection in accordance with a protocol approved by the Institutional Review Board and Research Committee of the University of Nebraska Medical Center (Omaha, NE). Isolation and culture of aortic SMC was established by using previously described techniques (9, 22). SMC were plated on collagen-coated 12-well plates at 1×10^5 cells/well. Cell cultures were treated with or without hIFN-γ at a concentration of 0.1 and 1.0 ng/ml in 1 ml/well of M-199 medium for 24 h. The medium was collected and spun at $3000 \times g$ at 4°C for 30 min to remove cell debris.

Gelatin zymography

Aortic proteins were extracted as previously described (9). The protein concentration for aortic proteins and cultured media from peritoneal macrophages and aortic SMC treated with or without IFN-γ was standardized with Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Gelatin zymography was performed as described previously by Longo et al. (8), with 0.8% gelatin in a 10% SDS-polyacrylamide gel. The molecular sizes of gelatinolytic activities were determined using protein standards (Bio-Rad).

Statistical analysis

Measurements of aortic diameter are expressed as mean ± SEM. A paired Student's *t* test was used to compare original and final diameter. Statistical significance was accepted at a $p < 0.05$.

Results

CD4-deficient mice are resistant to aneurysm formation

A role for $CD4^+$ T lymphocytes in aneurysm development is implied, as $CD4^+$ T lymphocytes are the major component of the cellular infiltrates present in AAA. To study the role of the $CD4^+$

T cells in the development of AAA, we used CD4-deficient ($CD4^{-/-}$) mice to test their response to $CaCl_2$ aneurysm induction. Eight weeks after periaortic application of $CaCl_2$, there was no significant change in aortic diameter or histology in $CD4^{-/-}$ mice (Table I) which were similar to the NaCl-treated group. However, the control C57BL/6 WT mice showed a 45.8% increase ($p < 0.01$) in aortic diameter after $CaCl_2$ treatment (Table I). Connective tissue staining of aortic sections from these mice showed disruption and fragmentation of medial elastic fibers (Fig. 1*b*), while NaCl-treated controls show intact medial elastic lamellae (Fig. 1*a*). These data demonstrate that $CD4^+$ T lymphocytes have a central role in aneurysm development. We also examined the aortic tissues for the presence of T cells ($CD3^+$). $CaCl_2$-induced WT mice show T cell infiltration (Fig. 1*e* and Table II), while $CD4^{-/-}$ mouse aortic tissue showed no T cell infiltration (Table II).

IFN-γ deficiency prevents aneurysm formation and MMP up-regulation during inflammation in IFN-$\gamma^{-/-}$ mice

Based on results obtained with $CD4^{-/-}$ mice, we surmised the effects of the $CD4^+$ cells was to secrete cytokines that promoted the local inflammatory response. IFN-γ can up-regulate MMPs and is increased in AAA, implicating it in AAA pathogenesis (4, 21, 23, 24). To examine the role of IFN-γ in AAA development, IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice underwent $CaCl_2$ aneurysm induction. Aneurysm development was suppressed in IFN-γ-deficient mice (Fig. 2*a*). Histologic analysis revealed minimal damage to the medial elastic lamellae in those mice (Fig. 2*c*), which is similar to the NaCl-treated control (Fig. 2*b*). T cell infiltration in $CaCl_2$-treated IFN-$\gamma^{-/-}$ mice is significantly decreased compared with $CaCl_2$-induced WT mice (Table II). The expression levels of MMP-9 and -2 from aortic tissues were examined in WT, $CD4^{-/-}$, and IFN-$\gamma^{-/-}$ mice. Both CD4 and IFN-γ gene-targeted mice exhibited significantly lower production of MMP-2 and MMP-9 in the aorta compared with WT mice after $CaCl_2$ induction (Fig. 3, *lanes 1–6*). These observations support previous observations that MMP-2 and MMP-9 are functionally significant in the connective tissue degradation in AAA and also indicate a mechanism by which $CD4^+$ T cells and IFN-γ regulate matrix metabolism through production of MMPs.

Aneurysms can be reconstituted in the $CD4^{-/-}$ mice by IFN-γ injection or infusion of competent lymphocytes into IFN-$\gamma^{-/-}$ mice

Because $CD4^+$ cells produce an array of cytokines, we next attempted to determine the specific contribution of IFN-γ. Recombinant murine IFN-γ was administrated i.p. to $CD4^{-/-}$ mice on the day after $CaCl_2$ or NaCl treatment. $CD4^{-/-}$ mice received 50,000 U of rmIFN-γ twice a week for 3 consecutive weeks. Table III showed that administration of rmIFN-γ alone partially reconstituted the $CaCl_2$-induced aneurysm in CD4-deficient mice. Connective tissue staining of aortic sections for those mice showed

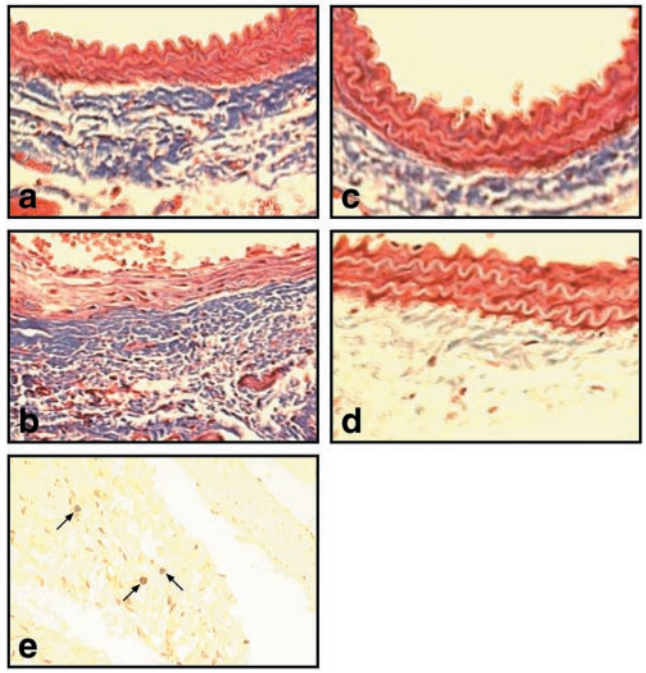

FIGURE 1. Aortic changes in WT and CD4-deficient mice responding to $CaCl_2$-aneurysm induction. *a-d*, Histological changes in mouse aorta by trichrome staining. NaCl-treated (*a*) and $CaCl_2$-treated (*b*) aorta from C57BL/6 mice; NaCl-treated (*c*) and $CaCl_2$-treated (*d*) aorta from $CD4^{-/-}$ mice. *e*, Immunohistochemical analysis of T lymphocyte infiltration in aortic tissue after $CaCl_2$ induction in C57BL/6. CD3-postive cells are indicated by arrows. Each staining represents three to five samples with similar results.

flattening of medial elastic fibers (Fig. 4*b*) compared with control (Fig. 4*a*). MMP-2 and -9 levels in aortic tissues of AAA were determined by zymography. The decreased production of MMP-2 and -9 in $CD4^{-/-}$ mice were restored by infusion of IFN-γ (Fig. 3, *lanes 7* and *8*). These results demonstrate that T cell cytokines, and IFN-γ in particular, are critically involved in AAA formation, in part, through their ability to regulate tissue-degrading enzymes (MMP-2 and -9) produced in the aortas. To confirm that IFN-γ from T cells alone was adequate to reconstitute aneurysm in IFN-$\gamma^{-/-}$ mice, we injected IFN-$\gamma^{-/-}$ mice with splenocytes from their corresponding WT background mice. This was done via a tail vein 1 day before calcium chloride aneurysm induction. A second splenocyte infusion was done after a week to booster cell number. IFN-$\gamma^{-/-}$ mice infused with WT splenocytes developed aneurysms that did not differ in size from competent WT mice treated with $CaCl_2$ (Table III). Histological analysis showed the typical elastin destruction following splenocyte infusion (Fig. 4*d*). Furthermore, T lymphocyte infiltration was similar to $CaCl_2$-induced

Table I. *Changes in aortic diameter in WT and $CD4^{-/-}$ mice after treatment of NaCl and $CaCl_2$*[a]

	C57BL/6		$CD4^{-/-}$	
Treatment	NaCl	$CaCl_2$	NaCl	$CaCl_2$
Number	10	10	10	10
Pretreatment (μm)	538 ± 8.2	532 ± 4.9	526 ± 9.1	534 ± 8.1
Posttreatment (μm)	548 ± 4.0	775 ± 6.9*	564 ± 7.0	580 ± 7.5
Percent of increase	3	45.8	7.4	8.7

[a] Aortic diameters were measured before NaCl or $CaCl_2$ incubation (pretreatment) and at sacrifice (posttreatment). Measurements of aortic diameter were expressed as mean ± SE. The percent increase was represented as a percent compared with pretreatment.

*, $p < 0.01$, Student's *t* test, compared to pretreatment value.

Table II. *CD3-positive cells found in aortic tissue from WT, CD4$^{-/-}$ and IFN-γ$^{-/-}$ mice after $CaCl_2$ aneurysm induction*[a]

	C57BL/6	CD4$^{-/-}$	IFN-γ$^{-/-}$
Number	4	4	4
T cell infiltrate	2 ± 0.8*	0 ± 0	0.75 ± 0.5

[a] CD3-positive cells in aortic adventitia and media were evaluated and scored with values from 0 to 3. Zero indicated that there were no CD3-positive cells; 1 indicated single cell might be T cells; 2 indicated one to three CD3-positive cells in a field; and 3 indicated more than three CD3-positive cells in a field. The values reflect the mean ± SE.

*, $p < 0.05$, Student's *t* test, compared to CD4$^{-/-}$ and IFN-γ$^{-/-}$ mice.

aneurysms in WT mice (Fig. 4*e*). To insure that the aneurysm formation did not represent a nonspecific response to splenocyte infusion, splenocytes harvested from CD4$^{-/-}$ mice were infused into IFN-γ$^{-/-}$ mice 1 day before and 1 wk after $CaCl_2$ aneurysm induction. There was no increase in aortic diameter or significant elastin destruction in mice infused with CD4$^{-/-}$ splenocytes (Table III and Fig. 4*c*). The partial reconstitution of AAA with IFN-γ infusion indicates that the products by CD4$^+$ cells are important in AAA formation. The partial aneurysm reconstitution by recombinant IFN-γ and full reconstitution by WT splenocytes infusion suggest that other cytokines from lymphocytes may also contribute to AAA formation or that i.p. IFN-γ dosing did not adequately replace intrinsic IFN-γ production. These results provide evidence that IFN-γ from T cells is critical for the development of calcium chloride-induced aneurysms.

IFN-γ induces productions of MMP-9 in macrophages and MMP-2 in SMC

To fully understand the role of IFN-γ in the regulation of monocyte/macrophage expression of MMP-9, peritoneal macrophages from C57BL/6 mice were isolated and cultured on polymerized collagen which mimics the physiological milieu of the arterial wall. Macrophages were treated with 0, 0.1, and 1.0 ng/ml of IFN-γ for 24 h. MMP-9 and MMP-2 secreted by macrophages was examined by gelatin zymography (Fig. 5*a*). We observed that IFN-γ stimulated the synthesis of MMP-9 and MMP-2. Only low levels of MMP-2 were produced by macrophages. To test the effect of IFN-γ on MMP-2 and MMP-9 expression in SMC, human aortic SMC were cultured and treated with 0, 0.1, and 1.0 ng/ml of IFN-γ for 24 h. Conditioned medium were analyzed by gelatin zymography (Fig. 5*b*). Production of MMP-2 was induced by IFN-γ treatment. No MMP-9 production in SMC was detected. These data suggest that IFN-γ induces production of MMP-9 from infiltrating macrophages and MMP-2 from local SMC. These observations combined with the findings of decreased MMP-2 and MMP-9 in the aorta of IFN-γ$^{-/-}$ and CD4$^{-/-}$ mice, support the hypothesis that IFN-γ from CD4$^+$ cells induces AAA by local up-regulation of two critical MMPs, MMP-2, and MMP-9.

Discussion

AAA involve disruption and attenuation of the elastic media and excessive production of matrix-degrading proteinases. The prominent inflammatory infiltrate has been implicated in the pathogenesis of AAA (16, 18). While studies of human tissues demonstrate the association between matrix destruction and inflammatory cells, animal data is even more compelling in demonstrating a causal role for local inflammation in AAA pathogenesis (8, 25, 26).

The inflammatory cell infiltrate in AAA is predominately composed of T lymphocytes and macrophages (18, 19). Elucidating specific contributions of subsets of inflammatory cells and their cytokines found in AAA will be one key to understanding AAA

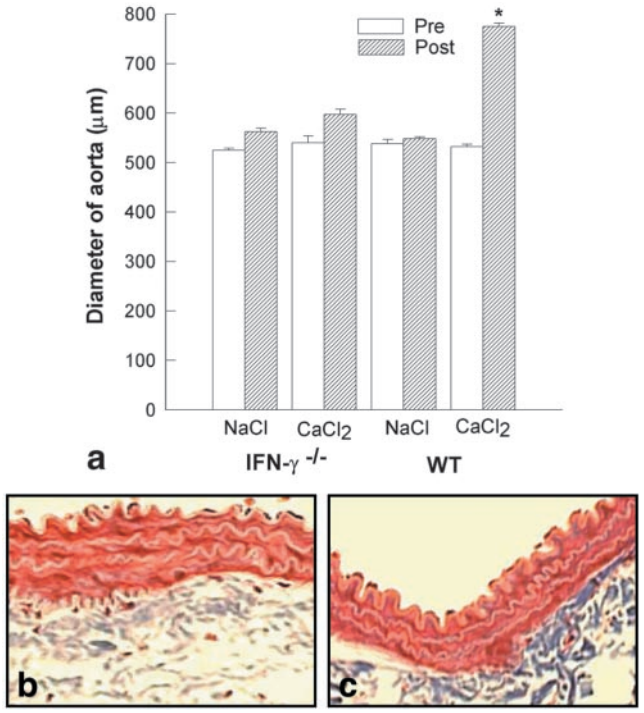

FIGURE 2. Effect of IFN-γ deficiency on the aneurysm development in $CaCl_2$-induced aneurysm model. *a*, Aortic diameter before (open bar) and 8 wk (hatched bar) after NaCl and $CaCl_2$ treatment in IFN-γ$^{-/-}$ mice. *b* and *c*, Histological changes in mouse aorta 8 wk after treatment using trichrome staining. NaCl-treated (*b*) and $CaCl_2$-treated (*c*) aorta from IFN-γ$^{-/-}$ mice. Each staining represents three to four samples with similar results.

and other chronic inflammatory disease processes associated with destruction of the extracellular matrix. CD4$^+$ T cells are the predominate immune subset in tissues from human AAA (18), suggesting a potentially important role in AAA formation. T cell infiltration is also one of the major characteristics in our murine aneurysm model (Fig. 1*e*). To understand the role of CD4$^+$ T cells in the pathogenesis of AAA, we used CD4-deficient mice, testing their response to $CaCl_2$ aneurysm induction. The hypothesis that tissue-infiltrating CD4$^+$ T cells play a central role in AAA development was confirmed by our finding that the absence of CD4$^+$ T cells prevents aneurysm development in a murine model. This aneurysm resistance is associated with decreased T cell infiltration. This is the first study to demonstrate that CD4$^+$ T cells are essential for aneurysmal degeneration. It is well-known that CD4$^+$ T

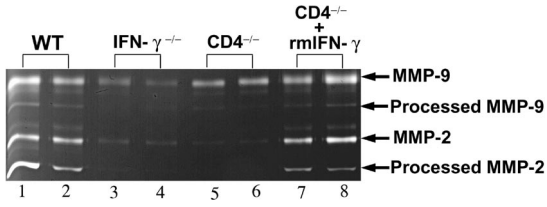

FIGURE 3. Gelatin zymographic analysis of MMP-2 and MMP-9 in mouse aorta after $CaCl_2$ aneurysm induction. Eight weeks after $CaCl_2$ treatment, mouse aortas were harvested. Aortic proteins were extracted and separated by electrophoresis on a 10% SDS-PAGE containing 0.8% gelatin. Gelatin zymography is representative of aortic protein extract from two samples in each group. *Lanes 1* and *2*, WT mice; *lanes 3* and *4*, IFN-γ$^{-/-}$ mice; *lanes 5* and *6*, CD4$^{-/-}$ mice; and *lanes 7* and *8*, CD4$^{-/-}$ mice treated with rmIFN-γ$^{-/-}$. The gel shown is representative of three trials with similar results.

Table III. *Changes in aortic diameter in CD4$^{-/-}$ and IFN-$\gamma^{-/-}$ mice with IFN-γ and splenocyte injection after $CaCl_2$ induction, respectively*[a]

	CD4$^{-/-}$		IFN-$\gamma^{-/-}$	
Induction	NaCl	$CaCl_2$	$CaCl_2$	$CaCl_2$
Treatment	IFN-γ	IFN-γ	CD4$^{-/-}$ T cells	CD4^{+} T cells
Number	8	8	5	5
Pre (μm)	472 ± 17	500 ± 11.9	526 ± 23.8	507 ± 14.2
Post (μm)	498 ± 21	670 ± 11.9*	546 ± 35.5	708 ± 9.9*
Percent of increase	5.4	34.1	3.4	39.6

[a] Aortic diameters were measured before $CaCl_2$ incubation (pre) and at sacrifice (post). Measurements of aortic diameter were expressed as mean ± SE. The percent increase was represented as a percent compared with pretreatment value.

*, $p < 0.05$, Student's t test, compared to pretreatment value.

cells are one of the major sources of IFN-γ (20). Furthermore, IFN-γ is a potent activator of macrophages and inducer of MMP-12 and cysteine proteinases (27). Importantly, circulating levels of IFN-γ are elevated in patients with AAA (21). In addition, Schönbeck et al. (28) have recently shown increased tissue levels of IFN-γ. Based on these observations, we hypothesized that IFN-γ was a pivotal mediator of AAA. To test this hypothesis, we injected CD4$^{-/-}$ mice with IFN-γ after aneurysmal induction. IFN-γ was able to reconstitute aneurysms in those mice. Our observations are consistent with those of Tillides et al. (29) who reported that IFN-γ can mediate atherosclerotic changes when T lymphocytes were absent. The observation that IFN-$\gamma^{-/-}$ mice are resistant to aneurysm formation lends further support to the concept that IFN-γ has a causal role in aneurysm formation. This effect was not a nonspecific because IL-6$^{-/-}$ mice developed aneurysms comparable to control mice. Despite this compelling evidence that IFN-γ is a pivotal mediator of AAA, we further demonstrated that competent splenocyte infusion, but not CD4$^{-/-}$ splenocyte, into IFN-$\gamma^{-/-}$ mice can reconstitute aneurysm formation in those mice. These results demonstrate that IFN-γ produced by CD4^{+} T cells is critical for aneurysm degeneration.

MMP-9, one of the most abundant elastolytic proteases secreted by human AAA tissues, is produced by aneurysm-infiltrating macrophage at the sites of tissue damage (14). Studies from our laboratory and other investigators (7) have shown that macrophage-derived MMP-9 is responsible for the local matrix destruction seen in experimental AAA. We have gone on to show the mesenchymal cell MMP-2 is also essential for aneurysm development in experimental animal models. To gain insight into the mechanisms of IFN-γ-mediated aneurysm, we determined whether IFN-γ altered the levels of expression of aortic MMPs. These studies demonstrate, for the first time, that elastic lamellar preservation in CD4$^{-/-}$ and IFN-$\gamma^{-/-}$ mice correlates with decreased local MMP-2 and MMP-9 expression. Aneurysm reconstitution in CD4$^{-/-}$ mice by IFN-γ infusion corresponds to restoration of MMP-2 and MMP-9 expression. This is consistent with the observation by Wang et al. (27), that IFN-γ stimulates the release of MMP-9 in vivo. Although several investigations have reported that IFN-γ down-regulates MMP-9 after it is maximally stimulated by Con A or mycobacterium (30) (31), we see evidence that IFN-γ regulates MMP-9 and MMP-2 both in vivo and in vitro. Macrophages and human SMC cultured on a collagen matrix designed to

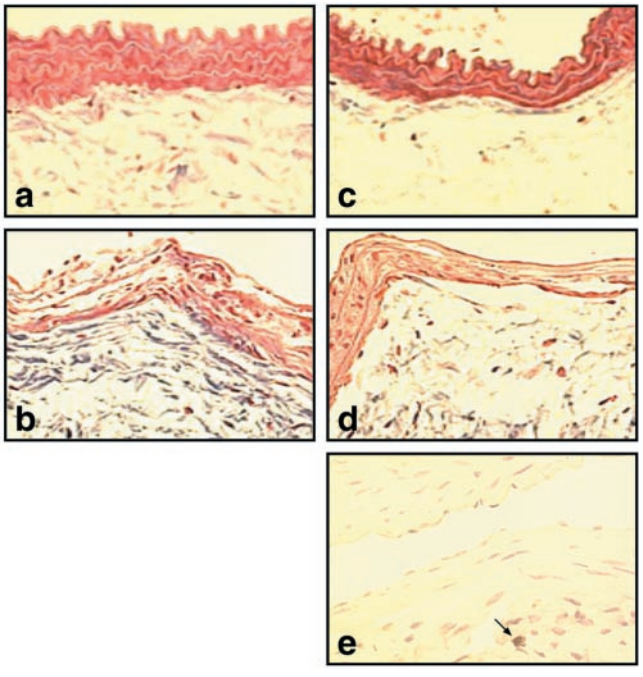

FIGURE 4. Aneurysm reconstitution by murine recombinant IFN-γ injection in CD4$^{-/-}$ mice and competent splenocyte infusion into IFN-$\gamma^{-/-}$ mice after $CaCl_2$-treatment. After NaCl or $CaCl_2$ treatment, CD4$^{-/-}$ mice were injected i.p. with 50,000 U IFN-γ, twice weekly for 3 wk; Following aneurysm induction, IFN-$\gamma^{-/-}$ mice were infused with 5×10^7 WT splenocytes through the tail vein twice weekly, or CD4$^{-/-}$ splenocytes for control. *a-d*, Histological analysis of aortic tissues. Cross-sections of the aortic tissues were stained for elastic fibers (trichrome staining). *a*, rmIFN-γ injected, NaCl-treated CD4$^{-/-}$ mice and *b*, rmIFN-γ injected, $CaCl_2$-treated CD4$^{-/-}$ mice; *c*, the IFN-$\gamma^{-/-}$ mice infused with CD4$^{-/-}$ splenocytes; and *d*, the IFN-$\gamma^{-/-}$ mice infused with WT splenocytes. *e*, Immunohistochemical analysis of T lymphocyte infiltration in aortic tissue after competent splenocyte infusion in IFN-$\gamma^{-/-}$ mice. CD3-postive cells are indicated by arrows. Each staining represents three experiments with similar results.

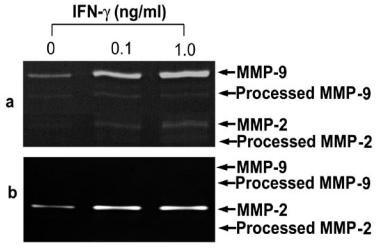

FIGURE 5. Up-regulation of the MMP-9 in macrophages and MMP-2 in SMC by IFN-γ treatment. *a*, Mouse peritoneal macrophages were cultured on collagen-coated plates and exposed to rmIFN-γ for 24 h at concentration of 0, 0.1, and 1.0 ng/ml. Conditioned media was separated on a 10% SDS-PAGE containing 0.8% gelatin for determination of MMP-9 content; *b*, human aortic SMC were cultured in M-199 medium and treated with hIFN-γ for 24 h at concentration of 0, 0.1, and 1.0 ng/ml. Conditioned media was separated on a 10% SDS-PAGE containing 0.8% gelatin for determination of MMP-2 content. The gel is representative of three experiments with similar results.

mimic the cells in the arterial wall exhibit increased MMP expression in response to IFN-γ. Tamai et al. (32) also observed that IFN-γ significantly induced the mRNA levels of interstitial collagenase and stromelysins in keratinocytes. Taken together, these data suggest that IFN-γ is a potent stimulator of MMP-2 and MMP-9. The findings demonstrate that the role of T lymphocytes in AAA is mediated largely by IFN-γ. $CD4^+$ lymphocytes promote degradation of the extracellular matrix of aortic wall through IFN-γ induced MMP expression. This study highlights a pivotal cytokine in the pathway of AAA formation that involves the IFN-γ signaling. It will provide a novel mechanism for understanding and treating AAA and other chronic inflammatory diseases.

References

1. Gregory, A. K., N. X. Yin, J. Capella, S. Xia, K. M. Newman, and M. D. Tilson. 1996. Features of autoimmunity in the abdominal aortic aneurysm. *Arch. Surg. 131:85*.
2. Newman, K. M., J. Jean-Claude, H. Li, J. V. Scholes, Y. Ogata, H. Nagase, and M. D. Tilson. 1994. Cellular localization of matrix metalloproteinases in the abdominal aortic aneurysm wall. *J. Vasc. Surg. 20:814*.
3. Nagashima, H., Y. Aoka, Y. Sakomura, A. Sakuta, S. Aomi, N. Ishizuka, N. Hagiwara, M. Kawana, and H. Kasanuki. 2002. A 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, cerivastatin, suppresses production of matrix metalloproteinase-9 in human abdominal aortic aneurysm wall. *J. Vasc. Surg. 36:158*.
4. Gerdes, N., G. K. Sukhova, P. Libby, R. S. Reynolds, J. L. Young, and U. Schonbeck. 2002. Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. *J. Exp. Med. 195:245*.
5. Rajavashisth, T. B., J. K. Liao, Z. S. Galis, S. Tripathi, U. Laufs, J. Tripathi, N. N. Chai, X. P. Xu, S. Jovinge, P. K. Shah, and P. Libby. 1999. Inflammatory cytokines and oxidized low density lipoproteins increase endothelial cell expression of membrane type 1-matrix metalloproteinase. *J. Biol. Chem. 274:11924*.
6. Davis, V. A., R. N. Persidskaia, L. M. Baca-Regen, N. Fiotti, B. G. Halloran, and B. T. Baxter. 2001. Cytokine pattern in aneurysmal and occlusive disease of the aorta. *J. Surg. Res. 101:152*.
7. Pyo, R., J. K. Lee, J. M. Shipley, J. A. Curci, D. Mao, S. J. Ziporin, T. L. Ennis, S. D. Shapiro, R. M. Senior, and R. W. Thompson. 2000. Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms. *J. Clin. Invest. 105:1641*.
8. Longo, G. M., W. Xiong, T. C. Greiner, Y. Zhao, N. Fiotti, and B. T. Baxter. 2002. Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J. Clin. Invest. 110:625*.
9. Davis, V., R. Persidskaia, L. Baca-Regen, Y. Itoh, H. Nagase, Y. Persidsky, A. Ghorpade, and B. T. Baxter. 1998. Matrix metalloproteinase-2 production and its binding to the matrix are increased in abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol. 18:1625*.
10. Carmeliet, P., L. Moons, R. Lijnen, M. Baes, V. Lemaitre, P. Tipping, A. Drew, Y. Eeckhout, S. Shapiro, F. Lupu, and D. Collen. 1997. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat. Genet. 17:439*.
11. Curci, J. A., S. Liao, M. D. Huffman, S. D. Shapiro, and R. W. Thompson. 1998. Expression and localization of macrophage elastase (matrix metalloproteinase-12) in abdominal aortic aneurysms. *J. Clin. Invest. 102:1900*.
12. Annabi, B., D. Shedid, P. Ghosn, R. L. Kenigsberg, R. R. Desrosiers, M. W. Bojanowski, E. Beaulieu, E. Nassif, R. Moumdjian, and R. Beliveau. 2002. Differential regulation of matrix metalloproteinase activities in abdominal aortic aneurysms. *J. Vasc. Surg. 35:539*.
13. Woessner, J. F., Jr. 1999. Matrix metalloproteinase inhibition: from the Jurassic to the third millennium. *Ann. NY Acad. Sci. 878:388*.
14. Thompson, R. W., D. R. Holmes, R. A. Mertens, S. Liao, M. D. Botney, R. P. Mecham, H. G. Welgus, and W. C. Parks. 1995. Production and localization of 92-kilodalton gelatinase in abdominal aortic aneurysms: an elastolytic metalloproteinase expressed by aneurysm-infiltrating macrophages. *J. Clin. Invest. 96:318*.
15. McMillan, W. D., N. A. Tamarina, M. Cipollone, D. A. Johnson, M. A. Parker, and W. H. Pearce. 1997. Size matters: the relationship between MMP-9 expression and aortic diameter. *Circulation 96:2228*.
16. Freestone, T., R. J. Turner, A. Coady, D. J. Higman, R. M. Greenhalgh, and J. T. Powell. 1995. Inflammation and matrix metalloproteinases in the enlarging abdominal aortic aneurysm. *Arterioscler. Thromb. Vasc. Biol. 15:1145*.
17. McMillan, W. D., B. K. Patterson, R. R. Keen, and W. H. Pearce. 1995. In situ localization and quantification of seventy-two-kilodalton type IV collagenase in aneurysmal, occlusive, and normal aorta. *J. Vasc. Surg. 22:295*.
18. Koch, A. E., G. K. Haines, R. J. Rizzo, J. A. Radosevich, R. M. Pope, P. G. Robinson, and W. H. Pearce. 1990. Human abdominal aortic aneurysms. Immunophenotypic analysis suggesting an immune-mediated response. *Am. J. Pathol. 137:1199*.
19. Pearce, W. H., and A. E. Koch. 1996. Cellular components and features of immune response in abdominal aortic aneurysms. *Ann. NY Acad. Sci. 800:175*.
20. Ito, S., P. Ansari, M. Sakatsume, H. Dickensheets, N. Vazquez, R. P. Donnelly, A. C. Larner, and D. S. Finbloom. 1999. Interleukin-10 inhibits expression of both interferon α- and interferon-γ-induced genes by suppressing tyrosine phosphorylation of STAT1. *Blood 93:1456*.
21. Juvonen, J., H. M. Surcel, J. Satta, A. M. Teppo, A. Bloigu, H. Syrjala, J. Airaksinen, M. Leinonen, P. Saikku, and T. Juvonen. 1997. Elevated circulating levels of inflammatory cytokines in patients with abdominal aortic aneurysm. *Arterioscler. Thromb. Vasc. Biol. 17:2843*.
22. Minion, D. J., Y. Wang, T. G. Lynch, I. J. Fox, G. D. Prorok, and B. T. Baxter. 1993. Soluble factors modulate changes in collagen gene expression in abdominal aortic aneurysms. *Surgery 114:252*.
23. Shin, K. Y., H. S. Moon, H. Y. Park, T. Y. Lee, Y. N. Woo, H. J. Kim, S. J. Lee, and G. Kong. 2000. Effects of tumor necrosis factor-α and interferon-γ on expressions of matrix metalloproteinase-2 and -9 in human bladder cancer cells. *Cancer Lett. 159:127*.
24. Anderson, F., B. A. Game, D. Atchley, M. Xu, M. F. Lopes-Virella, and Y. Huang. 2002. IFN-γ pretreatment augments immune complex-induced matrix metalloproteinase-1 expression in U937 histiocytes. *Clin. Immunol. 102:200*.
25. Anidjar, S., J. L. Salzmann, D. Gentric, P. Lagneau, J. P. Camilleri, and J. B. Michel. 1990. Elastase-induced experimental aneurysms in rats. *Circulation 82:973*.
26. Gertz, S. D., A. Kurgan, and D. Eisenberg. 1988. Aneurysm of the rabbit common carotid artery induced by periarterial application of calcium chloride in vivo. *J. Clin. Invest. 81:649*.
27. Wang, Z., T. Zheng, Z. Zhu, R. J. Homer, R. J. Riese, H. A. Chapman, Jr., S. D. Shapiro, and J. A. Elias. 2000. Interferon-γ induction of pulmonary emphysema in the adult murine lung. *J. Exp. Med. 192:1587*.
28. Schonbeck, U., G. K. Sukhova, N. Gerdes, and P. Libby. 2002. T(H)2 predominant immune responses prevail in human abdominal aortic aneurysm. *Am. J. Pathol. 161:499*.
29. Tellides, G., D. A. Tereb, N. C. Kirkiles-Smith, R. W. Kim, J. H. Wilson, J. S. Schechner, M. I. Lorber, and J. S. Pober. 2000. Interferon-γ elicits arteriosclerosis in the absence of leukocytes. *Nature 403:207*.
30. Wahl, L. M., and M. L. Corcoran. 1993. Regulation of monocyte/macrophage metalloproteinase production by cytokines. *J. Periodontol. 64:467*.
31. Quiding-Jarbrink, M., D. A. Smith, and G. J. Bancroft. 2001. Production of matrix metalloproteinases in response to mycobacterial infection. *Infect. Immun. 69:5661*.
32. Tamai, K., H. Ishikawa, A. Mauviel, and J. Uitto. 1995. Interferon-γ coordinately up-regulates matrix metalloprotease (MMP)-1 and MMP-3, but not tissue inhibitor of metalloproteases (TIMP), expression in cultured keratinocytes. *J. Invest. Dermatol. 104:384*.