T_H2 Predominant Immune Responses Prevail in Human Abdominal Aortic Aneurysm

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T lymphocytes localize within lesions of two diametrically opposed expressions of atherosclerosis: stenosis-producing plaques and ectasia-producing abdominal aortic aneurysm (AAA). T_H1 immune responses appear to predominate in human stenotic lesions. However, little information exists regarding the nature of the T-cell infiltrate in AAAs. We demonstrate here that AAAs predominantly express T_H2-associated cytokines and correspondingly lack mediators associated with the T_H1 response as determined by Western blot and immunohistochemical analysis. In particular, aneurysmal tissue expressed interleukin (IL)-4, IL-5, and IL-10, cytokines not or only faintly detected in nondiseased tissue or stenotic atheroma. In contrast, AAAs contained low levels of the T_H1 characteristic cytokines IL-2 and IL-15, which are amply expressed in stenotic lesions. Notably, stenotic lesions, but not AAAs, contained mature forms of the interferon- γ -inducing cytokines IL-12 and IL-18 as well as the IL-18-processing enzyme caspase-1. Moreover, aneurysmal tissue lacked the receptor for interferon- γ , although both types of lesions contained this T_H1-promoting cytokine. These findings suggest that the functional repertoire of T cells differs in stenotic and aneurysmal lesions, and provide a novel framework for understanding the mechanisms of these diametrically opposite expressions of atherosclerosis. (*Am J Pathol* 2002, 161:499–506)

In the United States alone abdominal aortic aneurysms (AAA) affect 3% of individuals 60 years or older, necessitate 46,000 surgical interventions, and cause ~15,000 deaths annually.¹ The prevalence of AAA will increase as the population ages and thus will continue to entail considerable morbidity, mortality, and medical expense. Despite considerable descriptive knowledge of the pathomorphology of AAA,^{2,3} insufficient understanding of the molecular mechanisms underlying its pathogenesis currently limits the prevention and treatment of this human disease.

No mechanistic model yet exists that explains why atherosclerosis can have diametrically opposed expres-

sions: aneurysm *versus* occlusive disease. Previous work by us and other investigators, however, revealed certain salient morphological characteristics in the pathophysiology of aortic aneurysm, including the profound inflammation characterized by abundant infiltrates of leukocytes such as T lymphocytes.^{4–7} Studies demonstrating that stiffness of the aneurysmal aortic wall does not correlate with the risk for rupture further supported the hypothesis that the inflammatory composition, rather than morphology or mechanics alone, determines the risk for acute clinical complications.⁸ Moreover, inflammatory infiltrates and aneurysmal enlargement correlate temporally, suggesting that the inflammatory cells may participate in the destruction of the aneurysmal aortic wall.⁹ Despite their abundance,^{4,6} the functional phenotype of the T lymphocytes found in AAAs remains mostly speculative.

T lymphocytes include both helper T cells (T_H) and cytolytic T cells. Within the helper subset, T_H1 cells secrete one characteristic set of cytokines [eg, interleukin (IL)-2, interferon (IFN)- γ , and lymphotoxin] and T_H2 cells secrete another, nonoverlapping set of cytokines (eg, IL-4, IL-5, IL-9, IL-10, IL-13).^{10,11} Among the prototypical T_H1 cytokines, IFN- γ has attracted particular interest, because this mediator activates macrophages; enhances inflammatory cell recruitment by augmenting cytokine, chemokine, and adhesion molecule expression; and amplifies immune responses in several ways, including increasing levels of MHC I and II molecules on antigen-presenting cells and endothelium. IFN- γ , synergistically induced by IL-12 and IL-18, accelerates experimental atherosclerosis.^{12–14} In contrast, T_H2-derived cytokines, in particular IL-4 and IL-10, tend to limit the cytotoxic potential of macrophages and to reduce the expression of proinflammatory mediators such as cytokines or matrix metalloproteinases (MMPs),^{15–19} enzymes that participate in the pathogenesis of atherosclerosis.^{20,21} Accordingly, a deficiency in IL-10 promotes experimental atherosclerosis.¹⁹ The cytokines produced by T_H1 or T_H2 cells tend to sustain their own development and antagonize each other.^{10,11} IFN- γ

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produced by T_H1 cells, for example, directly antagonizes T_H2 cells. IFN- γ also activates macrophages to produce more IL-12 and IL-18 (originally termed IGF, interferon gamma-inducing factor),^{22,23} which leads to the further expansion of T_H1 cells and inhibition of T_H2 cells. Conversely, IL-4 produced by T_H2 cells antagonizes the promotion of a T_H1 -directed immune response.

Recent studies from several groups, including our own, demonstrated a predominance of the T_H1 lymphocytic subpopulation and its mediators within atherosclerotic lesions.^{24–27} In particular, these studies demonstrated in atherosclerotic lesions expression of the T_H1 response-promoting cytokines IL-2, IL-12, IL-15, IL-18, CD40L, and IFN- γ , as well as the absence of the T_H2 cytokines IL-4, IL-5, and IL-10.

The present study tested the hypothesis that AAA and stenotic atheroma differ in the predominance of T_H2 -derived versus T_H1 -derived cytokines. Such distinction would provide novel molecular insight into potential pathways underlying these two diametrically opposed expressions of atherosclerosis.

Materials and Methods

Materials

Immunohistochemical and Western blot analysis used the following antibodies: mouse anti-human IL-2, IL-4, IL-5, IL-10, IL-15, and IL-18, as well as goat anti-human IL-18R α (R&D Systems, Minneapolis, MN); mouse anti-human IL-12_{p35} (Genzyme, Cambridge, MA); rabbit anti-human caspase-1_{p20} and CD40L (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-human IFN- γ and IFN- γ R α (Pharmingen, San Diego, CA); and mouse anti-human CD3 and CD68 (DAKO, Carpinteria, CA). To control for specificity a rabbit anti-human IL-4 and IL-10 antibody was used (Santa Cruz).

Surgical specimens of human carotid plaques were obtained from endarterectomies performed on eight different patients. Aneurysmal tissue was obtained as discarded material from AAA repair surgery on eight different patients. Nonatherosclerotic specimens were obtained from carotid arteries from autopsies ($n = 4$) and aortas from cardiac transplantation donors ($n = 3$). All specimens were obtained by protocols approved by the Human Investigation Review Committee at the Brigham and Women's Hospital. For the experiments, the fresh-frozen specimen were inspected visually and cut longitudinally into two equally diseased portions, which were applied in parallel to Western blot and immunohistochemical analysis, respectively.

Western Blot Analysis

Frozen nonatherosclerotic ($n = 7$), atheromatous human carotid ($n = 8$), or AAA specimens ($n = 8$) were homogenized (Ultra-turrax T 25; IKA-Labortechnik) and lysed (0.3 g tissue/ml lysis buffer: 10 mmol/L NaH₂PO₄, 150 mmol/L NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.2% NaN₃, 5

mmol/L ethylenediaminetetraacetic acid, 20 μ g/ml soybean trypsin inhibitor, 0.1 mmol/L phenylmethyl sulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin), as described previously.^{28,29} The lysates were clarified (16,000 $\times g$, 15 minutes) and the protein concentration for each tissue extract was determined using a bicinchoninic acid protein assay according to the instructions of the manufacturer (Pierce, Rockford, IL). Total protein (50 μ g) was separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) using a semidry blotting apparatus (3 mA/cm², 60 minutes; Bio-Rad). Blots were blocked, and primary and secondary antibodies were diluted in 5% defatted dry milk/phosphate-buffered saline (PBS)/0.1% Tween 20. Primary antibodies were applied in the following concentrations: mouse anti-human IL-2, IL-4, IL-5, IL-10, IL-15, IFN- γ , and goat anti-human IL-18R α at 2 μ g/ml; mouse anti-human IL-12_{p35}, IL-18, IFN- γ R α , and rabbit anti-human CD40L at 1 μ g/ml; and rabbit anti-human caspase-1_{p20} at 0.5 μ g/ml. After 1 hour of incubation with the primary antibody, blots were washed three times (PBS/0.1% Tween 20) and the respective secondary, peroxidase-conjugated antibody (Jackson ImmunoResearch, West Grove, PA) was added for another hour. Finally, the blots were washed (20 minutes in PBS/0.1% Tween 20) and immunoreactive proteins visualized using the Super-Signal West Femto kit (Pierce, Rockford, IL). Densitometric analysis of immunoreactive bands used GelPro software (Media Cybernetics, Silver Spring, MD), applied to digital images of the respective Western blots.

Immunohistochemistry

Serial cryostat sections (5 μ m) of surgical specimens of seven nonatherosclerotic carotids and aortas, eight atheromatous carotid plaques, and eight AAAs, were cut, air-dried onto microscope slides, fixed in acetone (-20°C , for 5 minutes), and preincubated with PBS containing 0.3% hydrogen peroxide to suppress endogenous peroxidase activity. Subsequently, sections were incubated (30 minutes) with primary [mouse anti-human IL-2, IL-4, IL-10 (all 1:50), IL-12_{p35}, IL-15 (both 1:100), IL-18 (1:200), IFN- γ R α (1:30), or rabbit anti-human caspase-1_{p20}, CD40L (both 1:100)] or control antibody, diluted in PBS supplemented with 5% appropriate serum. Staining was completed using the LSAB Kit (DAKO) according to the manufacturer's recommendations. In contrast, the goat anti-human IL-18R α (1:150) antibody was applied for 90 minutes, sections were incubated (45 minutes) with the respective biotinylated secondary antibody (Vector, Burlingame, CA), followed by avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector) and antibody binding was visualized with 3-amino-9-ethyl carbazole (Vector). Nuclei were counterstained by Gill's hematoxylin.

For co-localization of IL-4 with T lymphocytes and macrophages rabbit anti-human-IL-4 antibody (1:150) was applied for 90 minutes, followed by biotinylated secondary antibody (45 minutes) and Texas Red-conjugated

streptavidin (20 minutes; Amersham, Arlington Heights, IL). Subsequently, the avidin/biotin blocking kit (Vector) was applied, followed by overnight incubation (4°C) with mouse anti-human CD3 or CD68 antibodies. Finally, biotinylated horse anti-mouse IgG antibody was applied (45 minutes) followed by fluorescein isothiocyanate-conjugated streptavidin (Amersham).

Statistical Analysis

Data obtained from the densitometric analysis of immunoreactive bands are presented as fold regulation (mean $\pm$ SD) and were compared between atherosclerotic and aneurysmal tissue using the Student's *t*-test. A value of $P \leq 0.05$ was considered significant.

Results

To test the hypothesis that T_H1 or T_H2 responses predominate in the pathogenesis of human AAAs, we analyzed the expression of markers characteristic for either T-cell subset in human AAAs ($n = 8$) *in situ*. Observations on AAA tissue were compared with those on nondiseased ($n = 7$) tissue as well as atherosclerotic plaques ($n = 8$).

Interestingly, extracts of human AAA tissue displayed elevated expression of T_H2- and little or no expression of T_H1-associated cytokines. In particular, human AAA tissue contained the T_H2 type cytokines IL-4, IL-5, and IL-10, mediators not observed in extracts of nondiseased tissue (Figure 1). Notably, extracts of human atherosclerotic plaques also showed no immunoreactive IL-4 and, compared to AAA, much lower concentrations of IL-5 (7.4 ± 3.7 -fold, $P < 0.03$) and IL-10 (3.9 ± 2.1 -fold, $P < 0.05$); both immunoreactive bands (18 and 36 kD) were included in the quantification, because they presumably correspond to the monomeric and dimeric form of IL-10. In contrast, AAA tissue did not contain IL-2, a prototypical cytokine of the T_H1 subset, expressed abundantly in extracts of atherosclerotic plaques.^{27,30,31} Moreover, extracts of AAAs contained significantly less IL-15 (8.2 ± 5.5 -fold, $P < 0.01$) or CD40L (6.2 ± 4.8 -fold, $P < 0.05$), other characteristic T_H1 cytokines, compared to extracts of atherosclerotic plaques (Figure 1). Nonatherosclerotic tissue did not contain any detectable cytokines (Figure 1).

Immunohistochemical studies further supported the predominance of T_H2-associated cytokines and the absence of markers of the T_H1 subpopulation in human AAA. In accord with the Western blot analysis, AAA tissue showed enhanced staining for T_H2 cytokines such as IL-4 and IL-10 (Figure 2, right), and diminished or absent staining for characteristic T_H1 cytokines such as IL-2, IL-15, or CD40L (data not shown), compared to nondiseased tissue (Figure 2, left) or atherosclerotic plaques (Figure 2, middle). The T_H2 cytokines localized predominantly in T lymphocyte- as well as macrophage-enriched regions, as demonstrated by immunofluorescence double labeling for IL-4 with either CD3 or CD68, respectively (Figure 3).

Figure 1. Human AAAs contain T_H2-associated cytokines. Protein extracts (50 μ g/lane) of nondiseased carotids (Normal), atheromatous carotids, and AAAs were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent Western blot analysis using either a mouse anti-human IL-2, IL-4, IL-5, IL-10, IL-15, or CD40L antibody. The positions of the molecular weight markers are indicated on the left. Analysis of tissue extracts obtained from seven nonatherosclerotic, eight atherosclerotic, and eight AAA surgical specimens from different individuals showed similar results.

Because IFN- γ participates proximally in engendering T_H1 responses predominant in occlusive atheroma, and interruption of IFN- γ signaling markedly diminishes experimental occlusive atherosclerosis,^{12–14} we further analyzed the expression of this cytokine and its inducers IL-12 and IL-18 in human AAA. Extracts of nondiseased arterial tissue ($n = 7$) contained little or no IL-12 (Figure 4). In contrast, atheromatous tissue ($n = 8$) strongly expressed this cytokine when compared to extracts of AAA ($n = 8$) tissue (9.8 ± 2.5 -fold increase, $P < 0.01$; both, the 35- and 70-kD band were included in the quantification because they presumably correspond to the IL-12_{p35} subunit and the IL-12_{p70} dimer) (Figure 4). Moreover, AAA extracts expressed IL-18 predominantly as the inactive 24-kD precursor, whereas extracts of human atheroma contained predominantly the mature 18-kD form, in accord with previous observations.²⁹ In addition to the

Figure 2. Human AAAs express the characteristic T_H2 cytokines IL-4 and IL-10. Serial cryostat sections of frozen specimens of human nondiseased carotids (Normal), carotid atheroma, or AAA were stained with either mouse anti-human IL-4 or IL-10 antibody. The **asterisks** indicate the lumen of the vessel. The **inserts** show higher magnifications ($\times 400$) of the T cell/macrophage-enriched area. Analysis of tissue obtained from seven nonatherosclerotic, eight atherosclerotic, and eight AAA surgical specimens from different individuals showed similar results.

ligand, AAA specimens also expressed lower levels of the IL-18 receptor (IL-18 $R\alpha$) compared to atherosclerotic tissue (9.0 ± 2.2 -fold, $P > 0.01$) (Figure 4). In accord with the differential expression of mature IL-18, extracts of human atheroma, but not of AAA, contained immunoreactive bands corresponding to the active p20-subunit of the IL-18-converting enzyme, caspase-1 (Figure 4). In contrast, nondiseased arteries and aneurysmal specimens contained only the inactive caspase-1 precursor.

Although extracts of AAA contained less immunoreactive IFN- γ (2.8 ± 1.3 -fold, $P < 0.05$) than those obtained from stenotic atheroma (Figure 4), consistent with the diminished expression of the IFN- γ -inducing factors IL-12 and IL-18, this typical T_H1 cytokine localized in both types of diseased tissue. However, analysis of the expression of the receptor for IFN- γ (IFN- $\gamma R\alpha$) provided evidence for impaired IFN- γ -signaling in AAA. All nondiseased as well as AAA specimens analyzed lacked immunoreactive IFN- $\gamma R\alpha$, whereas extracts of human atheroma yielded immunoreactive bands in Western blot analysis (Figure 4). In accord with the biochemical data obtained with protein extracts, aneurysmal tissue exhibited diminished expression of the receptor for the IFN- γ -inducing factor IL-18, IL-18 $R\alpha$, and lack of the IFN- γ receptor *in situ*, as determined by immunohistochemical analysis (Figure 5).

Figure 3. The T_H2 cytokine IL-4 colocalizes with immunocompetent cells. Serial cryostat sections of frozen specimens of human AAAs were applied to immunofluorescence double labeling using anti-human IL-4 (**left**; red) as well as anti-human CD3 (**top right**; T lymphocytes, green), or CD68 (**bottom right**; macrophages, green) antibody. Analysis of three AAA surgical specimens from different individuals showed similar results.

Discussion

Risk factors for AAA and atheroma differ. Risk of atherosclerosis strongly correlates with the lipoprotein profile, the few eminent risk factors identified for aneurysmal disease include smoking and male gender.³² Interestingly, one of the major risk factors for human AAA, smoking, enhances the proliferation of the T-helper cell population,^{33,34} a predominant cell type of the aneurysmal inflammatory infiltrate. The balance between T_H1 - and T_H2 -type responses influences the progression of numerous inflammatory diseases, including atherosclerosis.^{18,27} The present study provides evidence that, in contrast to stenotic atherosclerotic plaques, human AAAs display a predominant T_H2 response, as determined by

Figure 4. Expression of IFN- γ -associated mediators is attenuated in human AAAs. Protein extracts (50 μ g/lane) of nondiseased arterial tissue (normal), atherosclerotic lesions, and AAA (specimens obtained from three different donors are shown) were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent Western blot analysis using either an anti-human IL-12_{p40}, IL-18, IL-18 receptor α (IL-18R α), caspase-1_{p20}, IFN- γ , or IFN- γ receptor- α (IFN- γ R α) antibody. The positions of the molecular weight markers are indicated on the left. Analysis of extracts of a total of seven nonatherosclerotic, eight atherosclerotic, and eight AAA specimens from different individuals showed similar results.

the accumulation of typical T_H2 cytokines and the absence or low expression levels of characteristic T_H1 cytokines, in particular the IFN- γ signaling pathway. This pattern of cytokine expression may reinforce itself, because the T_H2 lymphocyte subset not only promotes its own growth and differentiation by virtue of corresponding cytokine synthesis, but also suppresses the proliferation and differentiation of the T_H1 subset.^{10,11}

In particular, the T_H2 cytokine IL-4, overexpressed in AAA rather than human atheroma, not only promotes the proliferation of T lymphocytes but also specifically rescues T_H2, but not T_H1 cells, from apoptosis.³⁵ In contrast, IL-2, expressed in atheroma rather than AAA, prevents apoptosis of T_H1, but not of T_H2 cells.³⁵ In addition, the T_H2 cytokine IL-10 promotes the death of T_H1 cells.^{36,37}

Thus, elevated expression of IL-4 and IL-10 in combination with the low abundance of IL-2 and participants in the IFN- γ -signaling cascade, namely IL-12, IL-18, and IFN- γ (all T_H1-promoting cytokines), might foster the T_H2-dominated immune response in AAA. On the other hand, we and others recently demonstrated the *in vitro* and *in vivo* relevance for the characteristic T_H1 cytokines IL-12, IL-18, and IFN- γ in stenotic atheroma.^{12–14,29,38,39} Mice deficient for these cytokines have attenuated T_H1 activities.^{40,41} The lack of active IL-12, IL-18, IFN- γ , and their receptors in AAA supports our hypothesis that distinct T_H cell subsets predominate in occlusive atheroma and AAA. Of note, the impaired IFN- γ signaling observed in this study might explain the lack of IL-12, because IFN- γ augments both IL-12 production and expression of the IL-12 receptor, pathways typically promoting expansion of the T_H1 subset.^{42,43}

In addition to the numerical predominance of T_H2 cells, the cytokines overexpressed in AAA might directly modulate the pathological mechanisms underlying AAA. Aneurysms exhibit destruction of the normal arterial architecture, particularly the smooth muscle cell-enriched tunica media. Apoptosis of smooth muscle cells may contribute to aneurysm formation,^{6,44} dependent at least in part on Fas ligand (FasL) expressed on T lymphocytes.^{6,45} Interestingly, although both T_H1 and T_H2 lymphocytes express Fas and FasL, only the T_H2 subset expresses high levels of a Fas-associated phosphatase (FAP-1), which probably inhibits Fas signaling, causing death of T_H1 and selective survival of T_H2 lymphocytes in AAA.⁴⁶ Despite the lack of direct evidence that typical T_H2 cytokines can promote Fas-mediated apoptosis in smooth muscle cells, IL-4 and IL-10 sensitize nonlymphocytic cell types to Fas-mediated cell death⁴⁷ and can induce Fas resistance in B cells.⁴⁸ Interestingly, Fas-mediated apoptosis leads to the expression of IL-10 in lymphoid cells, suggesting a potential auto/paracrine feedback loop promoting apoptosis and the expression of T_H2 typical cytokines in AAA.⁴⁹

T_H2 cytokines might further promote the pathogenesis of AAA through another pathway, the degradation of extracellular matrix macromolecules such as collagen and/or elastin. Cytokines of the T_H2 subset augment collagenolytic and elastolytic activities in cell types associated with AAA. IL-4 and IL-10 elicit the expression of MMPs, such as the interstitial collagenase MMP-1 or MMP-3, in mononuclear phagocytes and smooth muscle cells,^{17,50} a function reversed in the presence of IL-1 β or tumor necrosis factor- α , cytokines that likely participate in atherosclerotic plaque formation but scant in AAA.^{51,52} Moreover, impaired IFN- γ signaling in AAA might heighten overexpression of matrix-degrading enzymes because this prototypical T_H1 cytokine potentially antagonizes IL-1 β - or tumor necrosis factor- α -induced MMP expression. Thus, the prevalence of T_H2 immune mediators in AAA might foster a pattern of matrix-degrading enzymes different from that observed in atherosclerotic plaques, in which a T_H1 immune response predominates.²⁷

In addition to collagen degradation, elastolysis figures prominently in the pathogenesis of AAA. Increased tissue

Figure 5. Expression of receptors for the T_H1 cytokines IL-18 and IFN- γ is diminished in human AAA. Serial cryostat sections of frozen specimens of nondiseased human arteries (Normal), carotid atheroma, or AAA were stained with antibodies recognizing either the human IL-18 receptor- α (IL-18R α) or IFN- γ receptor- α (IFN- γ R α). The asterisks indicate the lumen of the vessel. Analysis of tissues obtained from seven nonatherosclerotic, eight atherosclerotic, and eight AAA specimens from different individuals yielded similar results.

and serum levels of elastase activity and diminished elastin content compared to nondiseased or atherosclerotic plaque tissue characterizes AAA,⁵³ and neutrophil granulocytes probably contribute to the excessive elastolytic activity.⁵⁴ Chemoattraction of neutrophils by IL-4 and IL-5⁵⁵ might thus provide another pathway by which T_H2 cytokines could promote AAA formation. In addition, elastase released from neutrophils stimulates the expression of IL-10 by leukocytes,⁵⁶ potentially enabling operation of a positive feedback loop favoring elastolysis.

As suggested by the present data and as proposed by others, the pathogenesis of aneurysmal disease may involve elements distinct from usual atherosclerosis. Indeed, AAA and stenotic atherosclerotic plaques appear to represent diametrically opposed expressions of arterial disease involving distinct but overlapping risk factors, chronology, and pathogenic pathways. The present report provides evidence that a shift in the balance of T_H1 - and T_H2 -mediated immune responses, and thus the inflammatory response in the vessel wall, eventually characterizes these two diametrically opposed expressions of atherosclerosis, that encompass the two extreme cases of arterial remodeling.

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