High Prevalence of Circulating CD4\(^+\)CD28\(^-\) T-Cells in Patients With Small Abdominal Aortic Aneurysms

Christina Duftner, Rüdiger Seiler, Peter Klein-Weigel, Heike Göbel, Christian Goldberger, Christian Ihling, Gustav Fraedrich, Michael Schirmer

**Objective**—To assess the possible role of proinflammatory CD28\(^-\) T cells in abdominal aortic aneurysms (AAAs). Animal studies and human tissue studies suggest a role for interferon (IFN)-\(\gamma\)-producing T cells in the development and progression of AAAs.

**Methods and Results**—Fluorescence-activated cells sorter analysis of peripheral blood samples and measurement of AAA size using sonography were performed in 101 AAA patients and 38 healthy controls. Peripheral percentages of CD28\(^-\) T cells of the CD3\(^+\)CD4\(^+\) and the CD3\(^+\)CD8\(^+\) were enriched in AAA patients with 7.8\(\pm\)8.8\% and 41.9\(\pm\)15.7\% compared with healthy controls with 2.2\(\pm\)6.1\% and 24.9\(\pm\)15.5\%, respectively \((P=0.002\) and \(P<0.001\), respectively). Both CD4\(^+\)CD28\(^-\) and CD8\(^+\)CD28\(^-\) T cells produced large amounts of IFN-\(\gamma\) and perforin. Patients with small AAAs (<4 cm) showed higher peripheral levels of CD4\(^+\)CD28\(^-\) T cells than those with larger AAAs \((P=0.025\). Immunohistological examinations revealed 39.1\(\pm\)17.2\% CD4\(^+\)CD28\(^-\) and 44.0\(\pm\)13.8\% CD8\(^+\)CD28\(^-\) in AAA tissue specimens with inflammatory infiltrates.

**Conclusions**—IFN-\(\gamma\)- and perforin-producing CD28\(^-\) T cells are present in the periphery and the vessel wall of a majority of AAAs. This observation in humans favors the concept of a T cell–mediated pathophysiology of AAAs, especially during the early development of AAAs. (Arterioscler Thromb Vasc Biol. 2005;25:1347-1352.)

**Key Words:** aortic disease ■ aneurysms ■ leukocytes ■ immune system ■ human

Abdominal aortic aneurysm (AAA) is a common disease with a prevalence of 3\% of individuals aged 60 years and older and is a potentially lethal disorder causing \(\approx\)15 000 deaths annually in the United States.\(^1\)

Recent human tissue studies and animal models have led now to a paradigm shift in the pathogenetic concept of AAAs. Rather than a simple degenerative process, the majority of AAAs has proven to be a complex and dynamic remodeling process. In brief, studies of human AAA tissues have identified extensive inflammatory infiltrates in both the media and the adventitia,\(^2\) leading to an increased expression of proinflammatory cytokines and C-reactive protein (CRP) in aneurysmal tissue.\(^3-5\) The presence of vascular-associated lymphoid tissue (VALT) with lymphoid follicles and lymph node–like structures in the adventitia of AAAs suggests a role for immunocompetent and antigen presenting cells not only in atherosclerosis\(^6\) but also in AAA disease.\(^2\) The role for T cells has been further supported by immunogenetic findings with associations between AAA and human leukocyte antigen class II molecules.\(^7\) Infiltration of the adventitia usually occurs together with medial thinning. This inflammatory process triggers the production of metalloproteinases and apoptosis of medial smooth muscle cells thus explaining the disruption of the orderly lamellar structure in aortic aneurysms.

Recently, a subgroup of proinflammatory T cells has been identified in patients with immune-mediated disorders including rheumatoid arthritis,\(^8-9\) ankylosing spondylitis,\(^10\) multiple sclerosis,\(^11\) Wegener granulomatosis,\(^12\) and unstable angina,\(^13\) which lack the costimulatory molecule CD28 on their surface and are considered as markers for chronic inflammation and early aging.\(^14\) Under these circumstances the CD28\(^-\) T cells are part of the CD4\(^+\) as well as the CD8\(^+\) T cell compartment, persist over years, and include most of the oligoclonally expanded T cells. Phenotypically, CD4\(^+\)CD28\(^-\) T cells from rheumatoid arthritis and ankylosing spondylitis patients and CD8\(^+\)CD28\(^-\) T cells from aged persons, rheumatoid arthritis, and melanoma patients share the expression of various natural killer (NK) cell receptors and lack the expression of the lymphocyte marker CD7.\(^9,15-16\) Functionally these T cells are capable to release large amounts of interferon (IFN)-\(\gamma\), perforin, and granzyme B, providing them with the possibility to lyse target cells.\(^17\) In the pathogenesis of coronary arteriosclerosis, the critical role of IFN-\(\gamma\) was already investigated.

Original received November 12, 2004; final version accepted April 8, 2005.

From the Departments of Internal Medicine (C.D., C.G., M.S.) and Vascular Surgery (R.S., P.K.-W., G.F.), Innsbruck Medical University, Innsbruck, Austria; and the Department of Pathology (H.G., C.I.), University Hospital, Freiburg, Germany.

C.D. and R.S. contributed equally to this work and have to be regarded as co-first authors.

Correspondence to Michael Schirmer, MD, Assoc Prof, Clinical Department of Internal Medicine, Clinical Division of General Internal Medicine, Innsbruck Medical University, Anichstrasse 35, 6020 Innsbruck, Austria. E-mail michael.schirmer@uibk.ac.at

© 2005 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000167520.41436.c0

1347
in a mouse heart transplant model, and IFN-γ knockout recipients did not develop thickening of the arterial intima despite a T lymphocyte and macrophage infiltrate in the parenchyma.18

Therefore we examined in this study whether proinflammatory IFN-γ-producing CD4+CD28− and CD8+CD28− T cells would be enriched in patients with AAAs peripherally and locally. Additionally, the characteristics of these cells and a possible association between the percentages of CD28+ T cells and AAA size were studied.

Materials and Methods

Patients and Ethical Concerns

In a prospective design, all consecutive patients older than 50 and younger than 80 years of age with a diameter of the abdominal aorta larger than 3 cm were considered for recruitment into the study. Patients and controls with a history of neoplasm, recent acute infection, or history of any other immune-mediated chronic disease possibly related to elevated peripheral levels of CD28+ T cells were excluded from the study. In 49 consecutive AAA patients, a second blood draw was performed during follow-up. Maximal diameters of AAA were measured by sonography. Sonography was not performed in the controls. In our cohort 26.6% of AAA patients were treated by aneurysmectomy. The protocol was approved by the local ethics committee of the Medical Faculty of Innsbruck, Austria.

Immunostaining and Flow Cytometry

Surface staining of peripheral blood mononuclear cells (PBMCs) was performed using fluorescein isothiocyanate (FITC)-conjugated anti-CD4, anti-CD8, anti-CD7, anti-CD57, anti-CD45RO, anti-CD45RA, phycoerythrin-conjugated anti-CD28, and peridinin chlorophyll protein-conjugated anti-CD3, anti-CD4, or anti-CD8 monoclonal antibodies (all from Becton Dickinson, San Diego, Calif). For detection of apoptotic cells, cells were stained with Annexin V (Becton Dickinson). For intracellular staining of IFN-γ and perforin, stimulated cells were stained with FITC-conjugated anti–IFN-γ, anti-perforin, and control immunoglobulin, respectively (Becton Dickinson). For more information please see the expanded Methods (available online at http://atvb.ahajournals.org).

Immunohistochemistry

Serial sections were stained for CD4 (mouse anti-human monoclonal antibody; DAKO Cytomation, Denmark), CD8 (mouse anti-human monoclonal antibody; DAKO Cytomation), and CD28 (goat anti-human polyclonal antibody; R&D Systems Inc, Minneapolis, Minn) according to standard protocols using a three step avidin-biotin complex method.19 In brief, after fixation of the sections in acetone, incubation with the respective biotinylated secondary antibodies and avidin–biotin complex (SABC; DAKO Cytomation). The enzyme was developed with fast blue BB salt (Sigma) and HRP using the SPSS program, version 11.0. For more details please see the expanded Methods.

Prevalence of Circulating CD4+CD28− and CD8+CD28− T-Cells in AAA Patients

In the peripheral blood, percentages of CD28− of the CD3+CD4+ and the CD3+CD8+ cells were enriched to 7.8±8.8% and 41.9±15.7% in AAA patients compared with healthy controls with 2.2±6.1% and 24.9±15.5%, respectively (P=0.002 and P≤0.001, respectively, after adjustment for age and sex; Figure 1A). 95% of the age-matched healthy controls had CD3+CD4+CD28− T cells lower than 3.5% and CD3+CD8+CD28− T cells lower than 54.7%. Taking these levels as limits for normal accumulation of CD28− T cells,
60.4% of the AAA patients had elevated levels of CD4⁺CD28⁻ T cells and 19.8% showed increased levels of CD8⁺CD28⁻ T cells in the peripheral blood. Receiver operating curves (ROC) as a regression analysis model revealed comparable specificity and sensitivity of CD4⁺CD28⁻ and CD8⁺CD28⁻ T cells for AAA disease, with a calculated area under the curve (AUC) of 0.795 and 0.799, respectively. Using the two-sided Spearman-Rho test to analyze a possible dependency of circulating levels of CD4⁺CD28⁻ and CD8⁺CD28⁻ T cells, a correlation was found between these two T cell subsets (R=0.530, P<0.001).

The increase of peripheral levels of CD4⁺ and CD8⁺CD28⁻ T cells was independent of coexisting peripheral arterial occlusive disease (5.9±6.6% and 41.8±17.3%, respectively) or coronary heart disease (9.0±8.6% and 44.6±17.1%) compared with patients with AAs alone (7.5±9.2% and 39.7±13.9%, respectively).

Follow-Up and Apoptosis of Peripheral CD4⁺ and CD8⁺CD28⁻ T-Cells
During follow-up of 14.5±10.5 months, the levels of CD3⁺CD4⁺CD28⁻ T cells increased from 7.8±8.8% to 11.1±9.2% and levels of CD3⁺CD8⁺CD28⁻ T cells from 41.9±15.7% to 46.4±16.6% (n=49, P=0.006 and P=0.082, respectively). After staining with Annexin V, both CD4⁺ and CD8⁺CD28⁻ T cells showed reduced spontaneous apoptosis compared with their CD28⁺ T cell counterparts (3.3±1.8% and 4.2±2.7% to 19.2±9.2% and 17.7±9.9% Annexin V⁺ cells, P=0.007 and P=0.012, respectively).

Phenotypic and Functional Characterization of CD4⁺ and CD8⁺CD28⁻ T-Cells
For phenotypic characterization of CD28⁻ T cells, surface expression of CD7, CD57, CD45RA, and CD45RO were compared between CD28⁻ T cells and their CD28⁺ T cell counterparts (Figure 2A through 2H). The CD7 molecule, which is involved in T cell activation, is present on most normal human T cells under physiological conditions, but not on NK cells. CD7 surface expression was low on CD4⁺CD28⁻ T cells in comparison with CD4⁺CD28⁺ T cells (4.7±3.6% versus 52.6±18.0% positive cells, P=0.001; Figure 2A), whereas CD7 was less expressed on CD8⁺CD28⁻ T cells compared with their CD8⁺CD28⁺ T cell counterparts (67.2±19.1% versus 81.9±10.6% positive cells, P=0.020; Figure 2C). CD57 is a 110-kDa glycoprotein that is typically presented by NK cells. CD57 expression was increased on CD4⁺CD28⁻ T cells compared with CD4⁺CD28⁺ T cells (67.2±33.2% versus 2.5±3.2% positive cells, P=0.001; Figure 2B). CD57 was expressed on 70.7±10.7% of CD8⁺CD28⁻ T cells compared with 4.2±1.8% of CD8⁺CD28⁺ T cells (P<0.001; Figure 2D). CD45RA, the marker for naïve T cells, was expressed on 53.4±26.4% of CD4⁺CD28⁻ T cells and on 19.7±10.4% of CD4⁺CD28⁺ T cells (P=0.010; Figure 2E). On CD8⁺CD28⁻ T cells, surface expression of CD45RA was 72.7±14.8% compared with 20.8±16.4% on CD8⁺CD28⁺ T cells (P<0.001; Figure 2G). CD45 RO, the marker for memory T cells, was expressed on 42.4±24.1% of CD4⁺CD28⁻ T cells and 72.6±10.9% of CD4⁺CD28⁺ T cells (P=0.009; Figure 2F). On CD8⁺CD28⁻ T cells, surface expression of CD45RO was 10.9±10.8% compared with 59.2±17.3% on CD8⁺CD28⁺ T cells (P=0.001; Figure 2H). Representative FACS plots are shown as an example for phenotypic expression of CD7, CD57, CD45RA and CD45RO in Figure 1 (available online at http://atvb.ahajournals.org).

From the functional perspective, production of IFN-γ was more frequent in CD4⁺CD28⁻ than in CD4⁺CD28⁺ T cells (34.5±20.0% versus 14.6±8.1% IFN-γ-producing cells, P=0.003; Figure 3A) and more frequent in CD8⁺CD28⁻ than in CD8⁺CD28⁺ T cells (32.3±41.8% versus 14.5±25.1% IFN-γ-producing cells, P>0.05; Figure 3D). Interleukin
(IL)-4-producing cells were low in both CD4+CD28- and CD4+CD28+ T cells (1.9±1.5% versus 1.2±1.0% IL-4-producing cells, P>0.05; Figure 3B) and in CD8+CD28- and CD8+CD28+ T cells (2.9±2.0% versus 1.8±1.6% IL-4-producing cells, P>0.05; Figure 3E). Perforin-producing cells, however, were more frequent in CD4+CD28- than in CD4+CD28+ T cells (38.6±21.3% versus 1.6±1.1% perforin-positive cells, P=0.004; Figure 3C) and more frequent in CD8+CD28- than in CD8+CD28+ T cell subsets (49.0±26.4% versus 4.6±4.4% perforin-positive cells, P=0.043; Figure 3F).

Aneurysm Dimension and CD4+ and CD8+CD28- T-Cells

To test a possible association between the peripheral levels of CD4+ and CD8+CD28- T cells and the size of AAAs, maximal AAA diameters were subdivided into 3 groups: small (<4 cm, n=17), intermediate (4 to 6 cm, n=56), and large AAAs (>6 cm, n=17). Patients with small AAAs (<4 cm) showed higher peripheral levels of CD4+CD28- T cells (11.7±9.0%) than those with intermediate (6.6±7.7%, P=0.025) and large AAAs (6.7±5.6%, P=0.065; Figure 4A). No correlation was detected between the peripheral levels of CD8+CD28- T cells and the maximal AAA diameter (Figure 4B). Levels of CD4+ and CD8+CD28- T cells did not correlate with age, CRP, or ESR.

CD4+ and CD8+CD28- T-Cells in AAA

Tissue Sections

For immunohistological studies, single and double staining of CD4, CD8, and CD28 were performed to examine the local presence of these CD4+ and CD8+CD28- T cells in cryo-frozen tissue specimens from AAA patients who underwent surgery. Of 13 specimens from individual AAA patients, 7 specimens showed inflammatory infiltrations with lymphocytes predominantly in the outer part of the media and the adventitia (Figure 1B) and were used for further double staining evaluations. In the other specimens the media consisted of scar tissue with calcifications or missing adventitial tissue and immunohistochemistry showing only few scattered inflammatory cells and single smooth muscle cells. The ratio between CD4+ and CD8+ T cells within the lymphocytic aggregates was calculated to be 3.9±2.7. In our tissue specimens with inflammatory infiltrates, the levels of CD4+CD28- and CD8+CD28- T cells were 39.1±17.2% and 44.0±13.8%, respectively (Figure 1C). Although not significant, CD4+CD28- T cells were more frequent in the tissue (23.4±26.1%) than the peripheral blood (8.9±14.1%), whereas CD8+CD28- T cells appeared less frequent in tissue specimens (23.4±25.1%) than in the peripheral blood (44.0±15.0%, NS).

Discussion

The present study shows for the first time that peripheral percentages of CD28- of the CD3+CD4+ and the CD3+CD8+ T cells are expanded in the peripheral blood of AAA patients even without history or clinical signs of any other immune-mediated diseases. This observation further supports the concept of chronic inflammation including T cells in the pathophysiology of AAAs, and further underlines the systemic nature of many AAAs. Both CD4+ and CD8+CD28- T cells in the peripheral blood showed reduced spontaneous apoptosis compared with their normal CD28+ counterpart and persisted in the peripheral blood over months. This observation could explain the perpetuation of AAA disease after an initial trigger as proposed for rheumatoid arthritis.21

The crucial role for IFN-γ-producing T cells in the development of AAAs has been shown in a recent animal study with CD4+ and IFN-γ knock-out mice demonstrating the necessity of IFN-γ or IFN-γ-producing lymphocytes for the development of AAAs.22 Also in the development of atherosclerosis IFN-γ was shown to play an essential role in animal models, and immunofluorescent studies of human carotid specimens revealed activated T cells as a possible source of IFN-γ in the atherosclerotic plaques.23–25 Indeed, human CD28- T cells produce IFN-γ which then activates macrophages to produce proinflammatory cytokines and results in oxidative damage with lipid peroxidation attacking smooth muscle cells and matrix components of the vessel wall. Thus the disease process is perpetuated and cytotoxic features result in thinning of the arterial wall and aneurysm formation. Besides, both CD4+ and CD8+CD28- T cells release large amounts of perforin, and thus have to be considered as cytotoxic. Cytotoxic T cells are suspected to participate in tissue injury, and in vitro experiments already showed effective killing of endothelial cells by cytotoxic CD4+CD28- T cells.17 Recently a Th2-predominant inflammation and lack of IFN-γ signaling in the host were reported to induce murine aneurysms in allografted aortas.26 The IFN-γ receptor knockout mice show increased levels of IL-4 with markedly increased levels of matrix metalloproteinase (MMP)-9 and MMP-12. We wonder whether the concomitant development of a Th1 response as indicated by the prominent increase in IFN-γ expression might still have an effect on cells within the aortic graft and thus could trigger aneurysm formation.27 In our experiments both CD4+CD28- and CD8+CD28- T cells lack the intracellular production of IL-4 analyzed by FACS after stimulation (Figure 3B and 3E).

![Figure 4](image-url)

*Figure 4.* Aneurysm dimension (diameter measured by sonography) and peripheral levels of CD4+ and CD8+CD28- T cells. A. Patients with small AAAs (<4 cm) show highest peripheral levels of CD4+CD28- T cells. B. Peripheral levels of CD8+CD28- T cells do not depend on AAA diameter. Whiskers box plots show 50% of cases within the boxes and all data excluding mavericks between the end points of the whiskers (lines). Probability values were calculated using the Kruskal-Wallis test to compare small, medium, and large-sized AAAs, with P<0.05 considered as significant.
Indeed we found that these specific T cells were higher in the smaller AAAs (<4 cm) than in larger AAAs (≥4 cm). The correlation between CD28 T cells and the size of the AAAs parallels the findings by Hamano et al that tumor necrosis factor alpha (TNF-α) and macrophages are more present in small-sized AAAs than in large-sized AAAs.\(^{5}\) Taken together it appears that in the development of AAA the immune-mediated process occurs earlier in the disease with involvement of proinflammatory T cells, whereas the larger AAAs represent more an advanced stage with a minor role of immune-mediated processes. Thus in the early phase of AAA development the risk of AAA rupture is low,\(^{28–29}\) but the T cell–mediated process leads to macrophage activation with TNF-α production and subsequent elastin and collagen degradation and thus precedes the crucial changes of the AAA wall with potential rupture of the AAA in the later phase.

An increased prevalence of CD28 T cells had been proposed for several diseases including unstable angina and vasculitic diseases.\(^{12–13}\) Therefore we had excluded patients with such diseases, and had tested for possible correlation between CD4/CD28 T cells and other atherosclerotic diseases including peripheral arterial occlusive disease and coronary heart disease in the AAA patients. As coexisting atherosclerotic diseases had no influence on the levels of the CD28 T cells in AAA patients, the relationship between CD4/CD28 T cells and adventitial inflammation in the AAAs becomes even more obvious. This finding is also in line with the report that CD4/CD28 T cells had been shown to be lower in stable coronary heart disease than in unstable disease.\(^{13}\) The higher levels of CD4/CD28 T cells in smaller AAAs with more macrophages and higher levels of TNF-α compared with larger AAAs further support the concept of CD4/CD28 T cells correlating with AAA disease activity more than with atherosclerotic disease. As shown by others, exposure to TNF-α results in downregulation of CD28 in vitro, which may explain the higher prevalences of CD4/CD28 T cells in smaller AAAs.\(^{30}\) At present we can only speculate about a possible role of IL-12, which can only reverse downregulation of CD28 in vitro.\(^{31}\) The levels of IL-12 in larger AAAs have not been investigated so far.

The definite antigen recognized by the CD28 T cells remains unclear. Several studies have been performed to define possible triggers of AAAs, but results are still not conclusive.\(^{32–34}\) T lymphocyte expression of isoforms of CD45 corresponds with their ability to respond to recall antigens. The naïve T cell pool is defined by the marker CD45RA, whereas the responsive or memory T cell pool is defined by CD45RO.\(^{35–38}\) More recently, however, it has been shown that the CD45RA T cell population is heterogeneous, as CD45RO T cells can reconvert back to CD45RA T cells.\(^{37}\) In our AAA patients, both CD4+ and CD8+CD28 T cells revealed high percentages of CD45RA on the cell surface, which confirms their status as highly differentiated cells. Up to now, there is ongoing discussion regarding whether CD28 T cells are caused by chronic antigen stimulation or whether their characteristic phenotypic and functional properties is a result of premature senescence.\(^{38}\)

In the peripheral blood the levels of CD4+CD28 T cells correlated well with the levels of CD8+CD28 T cells. In the AAA tissue, however, CD4+CD28 T cells seemed to be enriched, whereas CD8+CD28 T cells appeared less frequent in the tissue specimens. Both cell types were stained and analyzed by the same technique using a computer assisted cell counting program. Therefore the CD4+CD28 T cells may be more important for the pathogenetic mechanisms of AAA than the CD8+CD28 T cells.

Our study has the limitation that AAA specimens were only available from those patients who underwent aneurysmectomy because of a large-sized AAA. Because CD28 T cells are less frequent in the large-sized AAAs, the immunohistological findings in this late phase of AAA may underestimate the number of immune-mediated AAAs. This could be explained by the fact that our surgeons take tissue specimens from AAAs from the mid-portion of the AAA, although more TNF-α mRNA and TNF-α–converting enzyme (TACE) mRNA had been described in the transition zone of AAAs.\(^{39}\) Thus the mid-portion of AAAs reflects more a non- or postinflammatory status, whereas a more active inflammatory status would be expected in the transition zone of AAAs. The source of biopsies could thus explain the lack of T cells in some of our specimens taken from the mid-portion of the AAAs.

In conclusion, these human data support a role for IFN-γ–producing cytotoxic CD28 T cells in the pathogenesis of a majority of AAAs as proposed by the CD4+ and IFN-γ–knock-out mouse model. T cell–mediated processes appear to be more important for initiation and initial development of AAA disease (<4 cm) but may lose importance in the larger AAAs after wall destabilization and disintegration.

**Acknowledgments**

The study was supported by the Research Fund of the Austrian National Bank (P 8835 and P 9715), Vienna, the Medical Research Fund of the Innsbruck Medical University (MFF), and the “Verein zur Förderung der Hämatologie, Onkologie und Immunologie,” Innsbruck, Austria.

**References**


High Prevalence of Circulating CD4^+CD28^- T-Cells in Patients With Small Abdominal Aortic Aneurysms

Christina Duftner, Rüdiger Seiler, Peter Klein-Weigel, Heike Göbel, Christian Goldberger, Christian Ihling, Gustav Fraedrich and Michael Schirmer

Arterioscler Thromb Vasc Biol. 2005;25:1347-1352; originally published online April 21, 2005; doi: 10.1161/01.ATV.0000167520.41436.c0

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/7/1347

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/04/22/01.ATV.0000167520.41436.c0.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
MATERIAL AND METHODS

Cell preparation, antibodies and 3-color immunofluorescence flow cytometry

Peripheral venous blood was drawn and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation.

Surface staining of PBMCs was performed using FITC conjugated anti-CD4, anti-CD8, anti-CD7, anti-CD57, anti-CD45RO, anti-CD45RA, phycoerythrin-conjugated anti-CD28 and peridinin chlorophyll protein-conjugated anti-CD3, anti-CD4 or anti-CD8 monoclonal antibodies (all from Becton Dickinson, San Diego, CA, USA). For detection of apoptotic cells, cells were stained with Annexin V (Becton Dickinson). For intracellular staining, cells were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate and 1 µg/ml ionomycin in the presence of 10 µg/ml brefeldin A for 4 hours (Sigma, Munich, Germany). After cell surface staining and permeabilization, cells were stained with FITC-conjugated anti-perforin and anti-IFN-γ and control immunoglobulin, respectively (Becton Dickinson). After fixation with 4 % paraformaldehyde cells were analyzed on a FACS-Calibur flow cytometer (Becton Dickinson). Gating was performed on CD4, CD8 and CD28, as appropriate. Thus, the intracellular production of cytokines could be directly compared between the CD28+ and CD28- T-cell cohorts. Data of these characteristics have been obtained from 5 to 10 experiments. Data were analyzed using WinMDI software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

Immunohistochemistry

Mid-portion transmural aortic tissue blocks of 1-1.5 cm length were obtained during regular surgery because of aneurysmectomy in AAAs with a diameter of 5.6 ± 1.0 cm and snap frozen in liquid nitrogen. Samples were cut in 5 µm thick sections, mounted on
SuperFrost slides and stored until further use at –80ºC. For lesion evaluation of cell typing and content one section was stained with HE. All lesions were classified according to the established histological classification of atherosclerosis,¹ and were classified as type IV or type V lesions. The media showed fibrosis and reduced numbers of vascular smooth muscle cells.

Serial sections were stained for CD4 (mouse anti-human monoclonal antibody, DAKOCytomation, Denmark), CD8 (mouse anti-human monoclonal antibody, DAKOCytomation) and CD28 (goat anti-human polyclonal antibody, R&D Systems Inc, Minneapolis, MN) according to standard protocols using a three step avidin-biotin complex method.² In brief, the sections were fixed for 10 minutes in acetone, and endogenous peroxidase was blocked by H₂O₂ in Tris buffered saline (TBS) and sodium azide for 20 minutes at room temperature. Nonspecific background was blocked by normal goat (DAKOCytomation) or normal donkey serum (JacksonImmunoResearch Lab. Inc.West Grove, PA, USA). After application of the primary antibody for 1 hour at room temperature in a wet chamber, the slides were incubated with the respective biotinylated secondary antibodies for 30 minutes at room temperature and eventually incubated with a horseradish-peroxidase (HRP)-labeled avidin-biotin-complex (SABC; DAKOCytomation) for another 30 minutes at room temperature. The enzyme was detected by 3-amino-9-ethyl-carbazole (AEC, Sigma) yielding a brownish-reddish color. Sections were counter-stained with hematoxyline. For double staining, donkey anti-mouse bound alkaline phosphatase was developed with fast blue BB salt (Sigma) and HRP with AEC. Sections were investigated under a light microscope (Axioscop, C.Zeiss, Jena).

CD4/CD8 ratio was calculated using a computer assisted cell counting program (analySIS 3.0 programme, Soft Imaging System GmbH Leinfelden-Echterdingen, Germany)
on double stainings. Five high power fields (HPF) of serial sections were evaluated per
analysis of lymphocytic aggregates in the adventitia.

**Statistics**

Values are expressed as mean ± standard deviation. Univariate variance analysis with
adjustment for the differences in age and sex between healthy controls and AAA patients was
used to compare peripheral levels of CD4⁺ and CD8⁺CD28⁻ T-cells in controls and AAA
patients. The two-sided Spearman-Rho test was used to analyse a possible correlation
between the peripheral levels of CD4⁺ and CD8⁺CD28⁻ T-cells, the paired t-test was used to
compare phenotypical and functional properties between CD28⁺ and CD28⁻ T-cell subsets
and regression analysis by receiver operating curves were performed to evaluate the
dependence on the CD28⁻ T-cells on AAA disease. For comparison of the peripheral levels
of CD4⁺ and CD8⁺CD28⁻ T-cells in patients with small, intermediate and large-sized AAAs
the Kruskal-Wallis test was used. All statistical analyses were performed using the SPSS
program, version 11.0 (Chicago, IL, USA). *P*<0.05 was considered as statistically
significant.

**REFERENCES**

1. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfeld
   ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of
   atherosclerotic lesions and a histological classification of atherosclerosis. A report
   from the Committee on Vascular Lesions of the Council on Arteriosclerosis,

2. Van der Loos CM, Das PK, Van den Oord JJ, Houthoff HJ. Multiple immunoenzyme
   staining techniques. Use of fluoresceinated, biotinylated and unlabelled monoclonal
**Table I.** Patients’ clinical characteristics.

<table>
<thead>
<tr>
<th>AAA risk factors</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>69.4±7.4</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>90/101 [89.1%]</td>
</tr>
<tr>
<td>hypertension (%)</td>
<td>48/90 [47.5%]</td>
</tr>
<tr>
<td>smoking (%)</td>
<td>34/90 [33.7%]</td>
</tr>
<tr>
<td>diabetes mellitus (%)</td>
<td>8/90 [7.9%]</td>
</tr>
<tr>
<td>hyperlipidaemie (%)</td>
<td>39/90 [38.6%]</td>
</tr>
<tr>
<td>coronary heart disease (%)</td>
<td>30/99 [30.3%]</td>
</tr>
<tr>
<td>peripheral artery occlusive disease (%)</td>
<td>23/99 [23.2%]</td>
</tr>
</tbody>
</table>
FIGURE LEGEND

**Figure I** Representative FACS plots as examples for phenotypical characterization of (A) CD4\(^+\) and (B) CD8\(^+\) subpopulations. Gating was performed on CD4\(^+\) and CD8\(^+\) lymphocytes for CD28\(^+\) and CD28\(^-\) compartments to directly compare between the corresponding subtypes. Histograms show percentages of positive cells (filled curves), with unspecific staining shown as overlay of immunoglobulin control assays (black line).
Figure I

**Panel A**
- CD4 PerCP: 77.3% (CD7), 52.6% (CD57), 5.9% (CD45RA), 19.7% (CD45RO)
- CD8 PerCP: 88.6% (CD7), 6.8% (CD57), 15.8% (CD45RA), 76.5% (CD45RO)

**Panel B**
- CD4 PerCP: 93.9% (CD7), 64.3% (CD57), 86.1% (CD45RA), 10.7% (CD45RO)
- CD8 PerCP: 93.9% (CD7), 64.3% (CD57), 86.1% (CD45RA), 10.7% (CD45RO)