Reduced Immunoregulatory CD31+ T Cells in Patients With Atherosclerotic Abdominal Aortic Aneurysm


**Background**—Cell-mediated immunity is considered to contribute to the pathogenesis of abdominal aortic aneurysms (AAA). In particular, infiltrating macrophages and CD8+ T lymphocytes participate in the destruction of the aortic wall extracellular matrix and smooth muscle cells. We surmise that these pathological events are controlled by circulating regulatory lymphocytes.

**Methods and Results**—Circulating CD4+/CD31+ cells were reduced in AAA patients (n=80, 8.9±0.6%) as compared with controls (n=69, 13.7±0.8%; P<0.001) and inversely proportional to AAA size. Exclusion of the aneurysm by an endoprosthesis did not affect CD31+ T cell values. Reduction of blood CD4+CD31+ cells was not attributable to their enrichment in AAA tissue. In contrast, CD8+CD31+ cells were slightly reduced in the blood while increased in the aneurysmal tissue (29.2±0.5 versus 20.2±4.7% in blood; n=6; P<0.05). Remarkably, high percentages of CD4+CD31+ cells were able to regulate proliferation and cytokine production of CD8+ lymphocytes, as well as CD8+ cell-mediated cytotoxicity of aortic smooth muscle cells (P<0.01). Finally, CD4+CD31+ cells reduced the production and activity of metalloproteinase-9 by lipopolysaccharide-stimulated macrophages.

**Conclusions**—Circulating CD4+CD31+ T cells regulate macrophage and CD8+ T cell activation and effector function in the arterial wall. Their reduction might promote the development of AAA. (Arterioscler Thromb Vasc Biol. 2006;26:618-623.)

**Key Words:** aortic diseases ■ immune system ■ blood cells
atory properties in vitro, may be critical in regulation of vascular immune-mediated pathologies such as atherosclerotic arterial aneurysms.

CD31 is a transmembrane glycoprotein present on the surface of endothelial cells, platelets, monocytes, granulocytes, and lymphocytes. Initially CD31 was classified as an adhesion molecule, however, it is now recognized that CD31 signaling results in cell inhibition. Among blood cells, only T cells lose CD31 in human adults. Lack of CD31 signaling allows enhanced T cell activation and a greater ability to infiltrate atherosclerotic arteries when they are likely to exert a pathogenic role. Our data show an enrichment of CD4/CD31+ T cells in the blood and the arterial tissues of AAA patients. These cells enhance the effector capacity of macrophages and of CD8+ T cells and can thus contribute to the pathogenesis of the disease. On the other hand, the presence of CD31 on the surface of circulating T cells likely allows not only their own constraint but also regulation of the interacting blood or vascular cells through homophilic CD31-CD31 inhibitory signaling. Here we report that AAA patients display a significant reduction in the percentage of circulating CD4/CD31+ T cells that may be responsible for an altered regulation of local macrophage and CD8+ T cell responses in the aneurysmal arterial wall.

Methods

Patients and Samples
Patients with a diameter of the abdominal aorta (measured by echography) larger than 3 cm (AAA, n=80) or apparently healthy subjects (n=32) or apparently healthy subjects (69) were either patients with essential peripheral arterial blood hypertension (n=32) or apparently healthy subjects (n=37). Subgroup analysis was performed according to the presence of hypertension and/or atherosclerosis (ie, the presence of carotid atherosclerotic plaques detected by high definition echotracking). Of the 80 AAA patients included in the study, 30 (37.5%) were patients with essential peripheral arterial blood hypertension (n=32) or apparently healthy subjects (n=37). Subgroup analysis was performed according to the presence of hypertension and/or atherosclerosis (ie, the presence of carotid atherosclerotic plaques detected by high definition echotracking). Exclusion criteria were: cancer, infection, and any other immune-mediated disease. The protocol was approved by the local ethical committee (CCPRPB Paris-Cochin n° 2095, n° 1930 and n° 1931) and informed consent was obtained from all subjects before sampling. Of the 80 AAA patients included in the study, 30 (37.5%) were on medical treatment only, 44 (55%) were treated by endoprosthesis placement, and 6 (7.5%) by aneurysctomy. In the latter (n=6), specimens of the anterior AAA wall were collected at the time of surgical intervention and were freshly treated with collagenase under optimal conditions to obtain cell suspensions for flow cytometry analysis. Cell viability of these cell suspensions was >90% as detected by propidium iodide positive cell exclusion by flow cytometry analysis. Heparinized blood samples were obtained in fasting conditions from all subjects and were immediately processed for the study. Age- and sex-matched Controls (n=69) were either patients with essential peripheral arterial blood hypertension (n=32) or apparently healthy subjects (n=37). Subgroup analysis was performed according to the presence of hypertension and/or atherosclerosis (ie, the presence of carotid atherosclerotic plaques detected by high definition echotracking). Exclusion criteria were: cancer, infection, and any other immune-mediated disease. The protocol was approved by the local ethical committee (CCPRPB Paris-Cochin n° 2095, n° 1930 and n° 1931) and informed consent was obtained from all subjects before sampling. Of the 80 AAA patients included in the study, 30 (37.5%) were on medical treatment only, 44 (55%) were treated by endoprosthesis placement, and 6 (7.5%) by aneurysctomy. In the latter (n=6), specimens of the anterior AAA wall were collected at the time of surgical intervention and were freshly treated with collagenase under optimal conditions to obtain cell suspensions for flow cytometry analysis. Cell viability of these cell suspensions was >90% as detected by propidium iodide positive cell exclusion by flow cytometry analysis. Heparinized blood samples were obtained in fasting conditions from all subjects and were immediately processed for the study.

Flow Cytometry
Blood leukocytes and tissue cell suspensions were incubated with a cocktail of 4 fluorescent monoclonal antibodies (BD Biosciences) directed to human CD25 (fluorescein isothiocyanate [FITC]), CD31 (PE), CD4 (PE-Cy5), and CD8 (allophtocyanin [APC]). A minimum of 2000 (AAA tissue) or 10 000 (blood) events in the lymphocyte gate (FSC-SSC) were acquired and analyzed on a FACS Caliibur (BD Biosciences).

Regulation of CD8+ Lymphocyte Activation by CD4+/CD31+ T Cells
CD8+ lymphocyte proliferation was elicited by mixed lymphocyte reaction using allogeneic dendritic cells (DC) as challenger. DC were generated from peripheral blood monocytes of a single healthy donor, as previously described. CD8+ and CD4+ lymphocytes were prepared from 6 healthy individuals on the same day and were isolated by negative selection (Dynal Biotech) rather than by positive sorting to avoid the potentially biasing effect of the cell-bound antibody on the subsequent functional assays. We had initially performed functional experiments using either total CD4+ T cells or CD4+/CD31+ and CD4-/CD31+ cell fractions obtained by positive fluorescent cell sorting. Significant differences were found between cultures containing total CD4+ as compared with those containing either CD4+/CD31+ or CD4-/CD31- T cells, whereas no difference was detected between CD4+/CD31+ and CD4-/CD31- positively selected cells. Indeed, the binding of the antibody to the CD31 at the surface of the CD4+ cells tended to inhibit the CD31-mediated interaction with the cognate CD8+ cells. Preincubation of total CD4+ T cells with the concentrations of anti-CD31 antibody equivalent to those used for positive cell sorting abolished the observed (regulatory) effect of total CD4+ T cells. Therefore, negative selection was used to deplete CD31+ cells from CD4+ lymphocyte preparations to be compared with total CD4+ T cells. A monoclonal mouse anti-human CD31 antibody (BD Biosciences) was added at 0.01, 0.1, and 1 µg/106 target (CD4+) cells to the antibody mix to obtain different percentages of CD31+ cells within CD4+ cell preparations. Purity of enriched CD8+ and CD4+ T cell populations was always >90% as assessed by flow cytometry. DC (106 cells per well), CD8+ (106 cells per well), and CD4+ (0.5×106 cells per well) cells were cocultured in complete RPMI 1640 containing 10% human male AB serum in 96-well plates. After 5 days, one fourth of cell culture supernatant was collected and frozen for subsequent ELISA measurement of IFN-γ and interleukin (IL)-2 (DuoSet, R&D system); and replaced with fresh complete medium containing 10 µL/mL of thymidine. Proliferation was evaluated after 16 hours in a β-counter. Either CD8+ or CD4+ cells were omitted in control cultures. CD4+ cell proliferation data were subtracted from CD8+ cell proliferation data and results are expressed as cpm (mean±SEM of triplicate values).

Regulation of CD8+ Cell-Mediated Cytotoxicity by CD4+/CD31+ T Cells
CD8+, CD4+, and CD31-depleted CD4+ cells were prepared as described above. Human SMC (ATCC-LGC) were labeled overnight with a lipophilic green fluorescent dye (DiO18 from the Live/Dead kit, #L7010; Molecular Probes) to be used as allogeneic target (T) cells (106 cells/well in 8-well glass Labtek culture plates). After two washes of the adherent SMC, CD8+ effector (E) cells were plated at 4×106 cells per well (E:T ratio=4:1) either alone or in the presence of total CD4+ or CD31-depleted CD4+ T cells (2×106 cells per well) and in the presence or absence of neutralizing antibodies for Fas-L (0.1 µg/mL, MAB126; R&D Systems) and IL-2 (0.1 µg/mL, MAB202; R&D Systems). SMC alone were used as control for spontaneous cytotoxicity. After 4 hours, the culture medium was collected for ELISA measurement (Fas-L, DuoSet; R&D system) and replaced by a buffer containing a low dose of propidium iodide (PI). Five minutes later, cells were detached and analyzed by flow cytometry according to the manufacturer’s instructions (Live/Dead kit; Molecular Probes). The percentage of cytosisysis (%CTL) was calculated as the number of PI+ target cells among total DiO18+ target cells. Duplicate cultures were cover-mounted (Vectashield-DAPI; Vector laboratories) and observed under fluorescent microscopy.

Regulation of Macrophage Activation by CD4+/CD31+ T Cells
Monocytes were enriched by negative magnetic selection (Dynal Biotech) from the peripheral blood of 3 healthy donors and cultured at 5×105 cells per well for 6 days in RPMI 1640 (10% fetal calf serum, 20 mmol/L HEPES, 2 mmol/L L-Glu, 50 ng/mL rHu M-colony stimulating factor [CSF]) in 8-well glass Labtek culture plates. On day 7, autologous total CD4+ or CD31-depleted CD4+ T cells prepared as above were added (2×105 cells per well) in the presence of fluorescence-quenched collagen type IV (100 µg/mL,
Oregon Green 488 conjugate, Molecular Probes), and of the reactive oxygen species sensor dihydroethidium (20 μmol/L; Molecular Probes) and in the presence or absence of LPS (10 ng/mL). After 3 hours incubation, MMP1/TIMP1 and MMP9/TIMP2 complexes (DuoSet; R&D Systems) were measured on the supernatant by ELISA. Adherent cells were thoroughly washed in PBS, detached, incubated with APC-conjugated anti-CD14 antibody, and analyzed by flow cytometry. Duplicate cultures were cover-mounted (Vectashield-DAPI) and observed under fluorescent microscopy.

Statistical Analysis
Results are expressed as Mean±SEM. Comparison of groups and experimental conditions were performed by ANOVA and correlation between variables by simple regression using Statview 5.0 software (SAS Institute Inc). Differences between groups were considered significant if P<0.05.

Results
CD31⁺ T Cells Are Reduced in AAA Patients
The number of total leukocytes, lymphocytes, granulocytes, and monocytes was similar in AAA patients and controls (Table I, available online at http://atvb.ahajournals.org). Similarly, no difference was found in the number and percentage of CD4⁺ and CD8⁺ lymphocytes, as well as that of CD4⁺/CD25⁺ cells (Table I). Four-color simultaneous staining allowed determining that >94% of CD4⁺/CD25⁺ cells were negative for the expression of CD31 at their surface (data not shown).

The number and percentage of circulating CD4⁺/CD31⁺ and CD8⁺/CD31⁺ lymphocytes was significantly decreased in AAA patients as compared with controls (Table I, and reciprocal changes were observed for the CD31⁺ fractions of CD4⁺ and CD8⁺ blood cells which increased in AAA patients as compared with controls (Table I). No difference was detected in CD31⁺ T lymphocyte percentages between patients eligible for endoprosthesis placement whether enrolled before (n=18) or after (n=26) the treatment (8.9±2.0 versus 7.4±0.7% of CD4⁺/CD31⁺ cells, NS; and 16.0±2.0 versus 18.7±1.3% of CD8⁺/CD31⁺ cells, NS).

The reduction of circulating CD4⁺/CD31⁺ cells in AAA patients was not dependent on the presence of either hypertension or atherosclerosis detected at the level of the carotid arteries (Table II, available online at http://atvb.ahajournals.org). Circulating CD8⁺/CD31⁺ lymphocyte reduction in the blood of AAA patients as compared with controls (Table I) was particularly evident when neither AAA patients nor controls showed hypertension (Table II). Linear regression analysis between the cross-section surface area of the aneurysm (in cm², calculated from the antero-posterior and latero-lateral diameter of the AAA, by echography) and the percentage of CD31⁺⁺ T lymphocytes showed that blood CD31⁺ T lymphocytes percentages were directly proportional to the AAA size, whereas an inverse correlation existed between blood CD31⁺ T cell percentages and AAA size. These correlations reached statistical significance in the case of CD4⁺/CD31⁺ and of CD8⁺/CD31⁺ cells (Figure 1) and not in the case of CD4⁺/CD31⁺ and of CD8⁺/CD31⁺ cells (data not shown).

CD8⁺/CD31⁺ But Not CD4⁺/CD31⁺ T Cells Are Sequestered in AAA Tissue
To evaluate whether the reduction of CD31⁺ T cells in the peripheral blood of AAA patients is a consequence of their sequestration in the aneurysmal wall where they can infiltrate, the percentages of CD8⁺/CD31⁺ and CD4⁺/CD31⁺ lymphocytes in blood were compared with those in autologous AAA tissue in 6 patients that were treated by aneurysmectomy. The percentage of CD8⁺/CD31⁺ lymphocytes was significantly increased in the AAA tissue as compared with the peripheral blood, whereas the percentage of CD4⁺/CD31⁺ lymphocytes reflected the data from circulating cells (Figure 2).

CD4⁺/CD31⁺ Cells Regulate CD8⁺ Lymphocyte and Macrophage Effector Function
DC-stimulated CD8⁺ T cell proliferation and cytokine (IFN-γ and IL-2) production in CD4⁺/CD8⁺ cocultures was increased, in a dose-dependent manner, as CD31⁺ (%CD4⁺ cells)
12% were CD31+/H11006 cells. Coculture of CD8 cells with CD4 total lymphocytes (of which 48.5±12% were CD31+) significantly reduced the extent of SMC cytosis (*P<0.01 vs CD8+/CD31− cells). Similarly, soluble Fas-L production (bottom) induced by allogeneic dendritic cell stimulation of SMC cytolyis (*P<0.05 vs lower dose; n.d. indicates not determined. Mean±SEM, n=6; *P<0.05 vs lower dose. Right, Spontaneous cytosis (CTL, top) of SMC in the absence of T cells was ~10%. The addition of CD8+ cells alone or in the presence of CD4+/CD31+ cells increased the percentage of SMC cytosis to a similar extent (~50%). Neutralizing antibodies for Fas-L (MAB126) abrogated more that 90% of CD8 T cell–mediated cytotoxicity and neutralizing antibodies for IL-2 (MAB202) reduced SMC cytotoxicity in CD8+/CD4+ CD31+ cocultures by 30%. Coculture of CD8 cells with CD4 total lymphocytes (of which 48.5±12% were CD31+) significantly reduced the extent of SMC cytosis (*P<0.01 vs CD8+/CD31− cells). Similarly, soluble Fas-L production (bottom) in the supernatant of these experiments was reduced in the presence of cocultured CD4 total cells as compared with the presence of CD4+CD31− cells or to SMC/CD8 cocultures devoid of CD4 cells. Mean±SEM, n=6; *P<0.05 vs lower dose; n.d. indicates not determined.

Figure 3. CD4+/CD31+ T cells regulate CD8+ T cell activation and cytotoxic function. Left, The presence of increasing numbers of CD31+ among CD4+ cells (0, 20, 40, or 60%) leads to significantly decreased CD8+ cell proliferation (top) and cytokine production (bottom) induced by allogeneic dendritic cell stimulation. Mean±SEM, n=6; *P<0.05 vs lower dose. Right, Spontaneous cytosis (CTL, top) of SMC in the absence of T cells was ~10%. The addition of CD8+ cells alone or in the presence of CD4+/CD31+ cells increased the percentage of SMC cytosis to a similar extent (~50%). Neutralizing antibodies for Fas-L (MAB126) abrogated more that 90% of CD8 T cell–mediated cytotoxicity and neutralizing antibodies for IL-2 (MAB202) reduced SMC cytotoxicity in CD8+/CD4+ CD31+ cocultures by 30%. Coculture of CD8 cells with CD4 total lymphocytes (of which 48.5±12% were CD31+) significantly reduced the extent of SMC cytosis (*P<0.01 vs CD8+/CD31− cells). Similarly, soluble Fas-L production (bottom) in the supernatant of these experiments was reduced in the presence of cocultured CD4 total cells as compared with the presence of CD4+CD31− cells or to SMC/CD8 cocultures devoid of CD4 cells. Mean±SEM, n=6; *P<0.05 vs lower dose; n.d. indicates not determined.

A growing body of evidence suggests that lymphocyte-mediated responses are involved in the pathogenesis of AAA,8,20–22 Locally infiltrating T cells produce inflammatory cytokines that in turn activate proteolysis of the extracellular matrix. These T cells also secrete death-promoting molecules such as perforin and Fas/FasL, that can mediate cytotoxicity of the resident cells and consequently reduce the local production of extracellular matrix components by these cells.5 Lack of appropriate immune regulation is responsible for immune-mediated self-tissue damages.9,21 The type of immune regulating cells varies according to the diversity of the diseased organ.10 In the present study, a significant reduction in the percentage of the circulating immunoregulatory CD4+CD31+ T cells was a hallmark of patients with AAA. The proportion of circulating CD4+/CD25+ T cells was not significantly different between patients and control subjects suggesting a crucial regulatory role in AAA for the CD31 itself, which is nearly absent at the surface of CD4+/CD25+ T cells.

CD4+/CD31+ circulating T cell reduction was not attributable to cell sequestration in the aneurysmal wall, because the percentage of CD4+/CD31+ T cells in the aneurysmal tissue was similar to that observed in the autologous circulating blood of the patients. Furthermore, CD4+/CD31+ T cell data were similar in patients whether evaluated before or after aneurysm exclusion by endoprosthesis placement. Therefore, the reduced number of CD4+/CD31+ cells is not an epiphenomenon but rather a primitive event in AAA disease and might possibly play a pathogenetic role.

CD31 is a self-interacting molecule expressed both on cells of the innermost layer of vascular wall and on leukocytes, and CD31-mediated inhibitory signals might be critical in regulation of vascular immune-mediated pathologies. Among human adult blood nucleated cells, T lymphocytes are the only circulating leukocytes to lose partially (CD8) or consistently (CD4) the expression of CD31 at their surface,13 and it has been previously demonstrated that CD4+/CD31+ T cells possess immunoregulatory properties.12 The regulatory role of both CD4+/CD25+ and CD4+/CD31+ T cells has been documented in CD4-mediated immune responses,9,12 but the role of CD4+/CD31+ T cells in the regulation of macrophages and CD8+ T cell-mediated immune responses remains unknown. We found that CD8+/CD31+ T cells are reduced in the blood and increased in AAA wall of patients suggesting that these cells are sequestered into the aneurysmal tissue where they could actively participate in the tissue damage by mediating cytosis of the smooth muscle cells, a key feature of the evolution of atherosclerosis toward aneurysm. Interestingly, the decrease in circulating CD8+/CD31+ T cells (and a fortiori their increase in AAA tissue) is directly proportional to the size of the aneurysm and therefore to the severity of the disease. Interestingly, the increased percentage of blood...
CD4⁺/CD31⁻ T cells in AAA patients is proportional to the extent of disease and the analysis of tissue-infiltrating lymphocytes suggests that these cells could play a pathogenic role in AAA pathology. Indeed, our in vitro data suggest that CD4⁺/CD31⁻ T cells may indirectly damage the aortic wall because they enhance CD8⁺ cell-mediated smooth muscle cell cytolysis and macrophage-derived MMP2/MMP9 activity.

CD8⁺/CD31⁻ T cells are also slightly increased in the blood of AAA patients, but circulating as well as tissue-infiltrating CD8⁺ cells are predominantly CD31⁺ and therefore a contribution by CD31⁻CD8⁺ T cells to the pathogenesis of AAA seems unlikely. In fact, sequestration of CD8⁺/CD31⁻ T cells within the lesions might explain the relative increase in CD8⁺/CD31⁻ T cells in the blood.

Unfortunately, the number of T lymphocytes and smooth muscle cells recoverable from AAA tissue is not sufficient to perform functional ex vivo experiments in patients. However, our data on commercially available aortic smooth muscle cells and blood donor–derived leukocytes show that CD8⁺ cell-mediated SMC cytolysis as well as CD8⁺ cell proliferation can be efficiently regulated by CD4⁺/CD31⁻ T cells, possibly via a modulation of the production of Fas-L by CD8⁺ T cells and of IL-2 by CD4⁺/CD31⁻ T cells. The presence of enriched CD4⁺/CD31⁻ T cells in CD8⁺/CD4⁺/SMC cocultures was also associated with an increased IFN-γ production. Human and as well as experimental aneurysm have been suggested to be associated with a Th2 environment. However, it has been shown that the Th1 cytokine IFN-γ alone is able to restore aneurysm formation in CD4 knockout mice, which are otherwise resistant to aneurysm formation in the CaCl₂ model.

Recent experimental studies suggest that the death of medial smooth muscle cells alone is not enough to cause aneurysm formation in allograft recipients. Local elastolytic and collagenolytic activity consistently contribute to AAA formation. In this perspective, our study points out a regulatory role for CD4⁺/CD31⁻ cells also on production and activity of macrophage MMP9 which is considered to be critical in the formation of AAA.

Together, our findings suggest that the reduction in the percentage of blood CD4⁺/CD31⁻ T cells observed in patients with atherosclerotic abdominal aneurysm might play an important role in the pathology because their reduction leads to an altered regulation of the cell-mediated immune responses involved in aneurysmal disease.

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Giuseppina Caligiuri, Patrick Rossignol, Pierre Julia, Emilie Groyer, Dikran Mouradian, Dominique Urbain, Namita Misra, Véronique Ollivier, Marc Sapoval, Pierre Boutouyrie, Srinivasa Krishna V. Kaveri, Antonino Nicoletti and Antoine Lafont

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