Atherosclerotic Abdominal Aortic Aneurysm and the Interaction Between Autologous Human Plaque-Derived Vascular Smooth Muscle Cells, Type 1 NKT, and Helper T Cells

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Abstract—Immune cell infiltration, vascular smooth muscle cell (VSMC) proliferation, and apoptosis are pathological hallmarks of atherosclerosis. The multifocal, chronic, and inflammatory nature of this disease of the cardiovascular system complicates targeted cellular therapy and emphasizes the need to understand the role and interaction of immune cells with VSMCs. We characterized the immune cell subsets present in human atherosclerotic tissue derived from atherosclerotic abdominal aortic aneurysm (AAA) and expanded them to study their interaction with autologous plaque-derived VSMCs in vitro. We show here that apart from T lymphocytes, plaque infiltrates consist of lots of NK cells and significant proportions of NKT cells that express T cell receptor (TCR) αβ, CD4, and the NK markers CD56 and CD161. The infiltrates are predominantly IFN-γ-producing Type 1 lymphoid cells. When cocultured, the T and NK cells adhere to VSMCs. CD4+ T cells enhance VSMC proliferation. VSMCs in turn enhance CD4+CD161+ NKT but not CD4+ or CD8+ T cell proliferation. CD4+CD161+ NKT cells inhibit VSMC proliferation by inducing apoptosis. Our results suggest that the interactions of Type 1 CD4+ T and CD4+CD161+ NKT cells with VSMCs may regulate VSMC proliferation and death respectively in atherosclerosis and the balance of these interactions could determine plaque stability. (Circ Res. 2005;96:675-683.)

Key Words: atherosclerotic abdominal aortic aneurysm ■ helper T cells ■ NKT ■ vascular smooth muscle cells ■ atherosclerosis

Atherosclerosis is an important chronic inflammatory disease of the vascular system.1,2 It is a slow dynamic progressive disease characterized by atherosclerotic lesions composed of dysfunctional endothelium, smooth muscle cells, lipid-laden macrophages, and T lymphocytes within walls of large elastic and muscular arteries.3 In addition to the immune response,4 the migration of vascular smooth muscle cells (VSMCs) into the intima of arterial walls and their focused proliferation in the intima is a key process of atherogenesis.5 VSMCs can also undergo apoptosis, resulting in plaque weakness.6,7 These processes can lead to a number of different vascular phenomena such as positive remodeling, vessel stenosis, aneurysm formation, or acute plaque rupture leading to thrombotic vessel occlusion. Atherosclerotic aneurysm, the common form of fusiform infrarenal human abdominal aortic aneurysms (AAAs), is usually accompanied by and developed from atherosclerotic plaques. The most prevalent features of atherosclerotic AAA include apoptosis of VSMCs and intense inflammatory cell (T lymphocytes and macrophages) infiltration of the aortic wall and increased cytokine and protease activity.8,9 Thus there is an imperative to understand the role and interaction of immune cells with VSMCs to allow targeted cellular therapy in this disease.

T lymphocytes, present in lesions at all stages of the disease, are a fundamental component of the histopathology of early and late lesions of atherosclerosis.1 They are more than an accidental bystander as many of these T cells express activation markers.10 In addition, there is evidence that plaque T cells are actively dividing11 and T cell cytokines like IFN-γ are expressed within atherosclerotic lesions of both human and mice.10,12 However, the types and distribution of T cell responses, and the mechanism by which they influence the disease process, are still not clear. Although FasL/Fas-mediated apoptosis of plaque-derived VSMCs have been investigated, such studies were conducted with monocytes derived from peripheral blood,13 with limited reflection on the...
cellular microenvironment of the plaque. Thus no studies have so far been performed to directly investigate the interaction of VSMCs with lymphoid cells (T and NKT), all plaque-derived. Such studies could be important in view that IFN-γ has been shown to potentiate Fas-mediated apoptosis and IFN-γ is a prominent proinflammatory mediator of atherosclerosis as evidenced by recent in vitro and in vivo studies that demonstrated that it could promote atherosclerosis. Whereas IFN-γ is largely produced by T lymphocytes, NK, and NKT cells, the precise role of these lymphoid cells, and what triggers their activation to IFN-γ production and subsequent proatherogenic activity, is unclear.

NKT cells represent a subset of mature unconventional T cells that is defined as expressing both the T cell receptor (TCR) αβ and markers characteristic of NK cells such as CD161 (NK1.1), CD122 (IL-2Rβ), and CD5620–22 and have distinctive phenotypic and functional properties. They have been extensively characterized in mice but are also present in human, and differences have been reported between the two species. The prototypic NKT cells express the invariant TCRα chain (Vα24JαQ in the human) and are restricted by the nonpolymorphic class 1 molecule, CD1d. They are mainly of the CD4+ or CD4 CD8- phenotype and can be detected wherever conventional T cells are found, although the proportion varies widely in a tissue-specific manner. Functionally, NKT cells exert potent cytolytic activity mediated by perforin/granzyme B and FasL. Both mouse and human NKT cells rapidly secrete cytokines associated with type 1 (IFN-γ, IL-2, TNF-α) or type 2 (IL-4, IL-5, IL-10, IL-13) responses on TCR engagement and reaction with glycolipid antigen. This apparent Th0 profile of cytokine production may be skewed toward a predominance of IFN-γ 25 or IL-4 26 to promote Type 1 responses under certain conditions and Type 2 responses in others, respectively. This coincides with their stable expression of IL-18R or ST2L in Plaques in AAA

Materials and Methods

Patient Specimens

Atherosclerotic tissues were obtained from patients with abdominal aneurysm undergoing elective surgery for graft repair of the infrarenal abdominal aorta. Fifteen patients, 12 men and 3 women, aged between 55 to 85 years old were included in the study. They are all vasculopathies with other features of atherosclerosis such as myocardial ischemia, peripheral vascular disease, cerebrovascular disease, or renal atherosclerotic impairment, suggesting an atherosclerotic as opposed to vasculitic or connective tissue etiology. The patients’ demographic profile is presented in online Table 1 (see the online data supplement). Most of the patients have at least three risk factors for atherosclerosis and are hypertensive, smokers, and male but not diabetic. The tissues were removed from the aortic sacs at the time of surgery, placed immediately in chilled RPMI 1640 medium, and processed within 24 hours. All the tissues were obtained with patient consent and local ethical approval.

For an expanded Materials and Methods section, see the online data supplement available at http://circres.ahajournals.org.

Results

Presence of Immune Infiltrates in Atherosclerotic Plaques in AAA

To demonstrate the presence of atherosclerotic plaques and to locate inflammatory cells in human AAA tissue, consecutive cryosections were stained with the histological stains hematoxylin and eosin, Verhoeff von Giessen, or Oil red O with hematoxylin (Figure 1a). We demonstrate in this study a section of atheroma tissue that contains an atherosclerotic plaque with a lipid core stained with oil red O (top right), and this is evident in all 15 patients. Furthermore, clumps of lymphoid infiltrates are found mainly in the media and intima surrounding the plaque and not in the adventitia (top left), suggesting that the lymphoid cells are involved with the atherosclerotic process. This is typical of other atherosclerotic AAA tissue sections tested. Characteristic of aneurysm tissue, there is increased neovascularization with reduced elastin in the media and increased collagen mass in the adventitia (bottom). The presence of immune infiltrates in the plaque and media is further supported by 3-color immunohistochemistry of this and other consecutive cryosections of the same tissue (Figure 1b). In the tissue section shown, there is an abundance of TCRαβ CD161+ NK (red cells, bottom left) and TCRαβ CD161+ NKT cells (yellowish green/ red cells, bottom left) in this clump of infiltrates near a region of neovascularization, and they are type 1 IFN-γ-producing cells (bottom right) as evidenced by their expression of IL-18R, a type 1 marker.27,29 These results indicate that apart from T lymphocytes, NK and NKT cells are present in very high proportions in atherosclerotic AAA tissue.

Predominance of Type 1 T, NK, and NKT Cells in Plaques

We have recently demonstrated that type 1 (IFN-γ producing) lymphoid cells stably express IL-18R, whereas type 2 (IL-4 producing) lymphoid cells stably express ST2L in mice and human.27–29 To assess the proportion of type 1 and type 2 lymphoid cells in the infiltrates of plaques, we performed 3-color flow cytometric analysis on ex vivo immune cells from 15 AAA tissues using antibodies to IL-18R or ST2L with antibodies to
appropriate combinations of the T cell markers TCRαβ, Vα24, CD3, or CD4 and the NK cell markers CD161 or CD56. Our data not only further support the presence of NK and NKT cells in immune infiltrates within human atherosclerotic aneurysmal tissue, but that there are higher proportions of type 1 CD4+ T helper (Th), NK, as well as the TCRαβ+CD56+/CD161+, CD3+CD56+/CD161+, and Vα24+CD56+/CD161+ NKT cells, compared with type 2 cells, in human atherosclerotic plaques (Figure 2). Being predominantly IL-18Rγ, these type 1 lymphoid cells are chiefly responsible for elaborating the central proatherogenic factor, IFN-γ. The proportion of invariant Vα24+ NKT is variable between tissues and may constitute only ~15% of total NKT. Furthermore, the proportion of different lymphoid cell subsets (T, NK, and NKT) can vary depending on the location of the infiltrate.

**Figure 1.** Stained cryosections of atherosclerotic plaque containing AAA tissue. a, Histological stainings of consecutive sections to demonstrate the distribution of lymphoid infiltrates, lipid core, elastin, and collagen in atherosclerotic aneurysm. Hematoxylin and eosin stained lesion (top left) show hematoxylin-stained clusters of infiltrates (arrows). Dense lipid droplets are stained red with Oil red O (arrows, top right). Clusters of hematoxylin stained infiltrates (dark blue) are located in the vicinity of the lipids. Verhoff von Giesson–stained section (bottom) shows reduced elastin fibers (black) in the media and increased collagen (dark red) mass in the adventitia. Original magnification ×20. b, NK and NKT cells in the plaque in a consecutive section of the same tissue shown by 3-color immunohistochemistry with merged image (red and green, bottom left). Type 1 NKT cells (yellowish green/red, bottom left) are found in abundance within immune infiltrates in this plaque section as TCRαβ+ lymphocytes (green, top right and bottom left), that are colabeled with the NK marker CD161 (red, top and bottom left) and the type 1 marker IL-18R (bottom right, blue). This is representative of plaque immunohistochemistry from four other donors. Original magnification ×400.

**Figure 2.** Predominance of ex vivo IFN-γ-producing type 1 Th, NKT, and NK cells in immune infiltrates in human atherosclerotic plaques. Three-color flow cytometric analysis of the distribution of ST2L+ vs IL-18Rγ+ T (n=15), NKT (n=15), and NK cells (n=13) obtained from 15 AAA tissue. NKT cells analyzed include the TCRαβ+CD56+/CD161+, CD3+CD56+/CD161+, and Vα24+CD56+/CD161+ subsets. Probability values were obtained using the Wilcoxon matched pairs signed ranks test.

**VSMCs Support NKT but not T Cell Growth**

To test our hypothesis for the involvement of NKT cells in atherosclerosis, plaque lymphoid cells (both T and NKT) were initially expanded in vitro with immobilized anti-CD3 and anti-CD161 or anti-CD28 as costimulators in the presence of autologous plaque VSMCs. The lymphoid cells and VSMCs proliferated well and expansion was enhanced by direct contact between lymphoid cells and VSMCs in the culture, as shown by large clumps of blasting lymphoid cells adhering tightly to VSMC fibers (Figure 3a). The observed lymphoid cell activation and proliferation was not due to allogenic response because autologous VSMCs and lymphoid cells were used. Adherence of both T and NKT cells was confirmed in cocultures of VSMCs with lymphoid cells and by 3-color immunostaining, which shows one blue-colored CD3+CD161+ T cell and a reddish blue CD3+CD161+ NKT cell adhering to the green α-actin containing VSMCs (Figure 3b through 3g). To further investigate the nature of the interaction between VSMCs and T or NKT cells, the lymphoid cells were further expanded with restimulation in the absence of VSMCs before positive selection for NKT and T cells. Before positive selection, the lymphoid cells were screened to estimate the proportion of CD4+CD56+/CD161αβTCR+ NKT cells available for selection. Because
≈65% of CD161αβTCR+ NKT and all CD161αβTCR+ T cells express CD4 (Figure 4a) and none of the CD4+CD161− NKT cells express a γδ TCR (Figure 4b), the lymphoid cells were then positively sorted into CD4+ or CD4+CD56+ CD161+ NKT cells and confirmed by flow cytometry before use. They were predominantly type 1 CD3+CD161+IL-18R− NKT or CD3+CD161−IL-18R+CD4+ Th cells (Figure 4c and 4d), respectively. VSMCs support of NKT cell growth was confirmed by monitoring, via 3H-thymidine incorporation, the proliferation of positively sorted CD3+CD4+CD161+ NKT cells in the absence or presence of fixed autologous VSMCs (Figure 5a). Such enhancement of NKT cell proliferation was dependent on direct contact with fixed VSMCs, as the effect was abrogated by pretreatment of fixed VSMCs with anti-VCAM or anti-CD40 but not with anti-ICAM or control normal mouse IgG. We next investigated whether, apart from direct contact, human VSMCs could also control normal mouse IgG. We next investigated whether, apart from direct contact with fixed VSMCs, as the effect was abrogated by pretreatment of fixed VSMCs with anti-VLA-4, anti-LFA-1, and anti-CD40L antibody. Interestingly, this process was contrasted by our demonstration in this study that NKT cells induced autologous VSMCs to apoptosis. This was demonstrated by the inhibition of VSMC proliferation in the presence of fixed NKT cells (Figure 6b). The inhibition was almost completely reversed by the presence of the broad-spectrum caspase inhibitor z-VAD-FMK, suggesting that NKT cells were inducing VSMC apoptosis. The induction of apoptosis in VSMCs was further confirmed by staining VSMCs, treated or not with fixed NKT cells, with annexin V-FITC and propidium iodide. In the presence z-YVAD-FMK, the inhibitor specific for caspase 1/ICE and caspase 4, VSMCs treated or not with fixed NKT cells, with annexin V-FITC and propidium iodide. In the presence z-YVAD-FMK, the inhibitor specific for caspase 1/ICE and caspase 4, the reversal (40%) of apoptosis was, however, less dramatic (Figure 6c). This suggests that some other caspase(s) may also be involved in the apoptotic process. In addition, when VSMCs were cultured in the presence of IFN-γ or spent NKT culture medium (SN) containing 788pg/mL IFN-γ, they showed a substantial increase (50%) in expression of Fas compared with in the presence of medium (med) alone (Figure 6d), indicating that IFN-γ produced by NKT1 cells could upregulate Fas expression on VSMCs. This could synergistically promote FasL-mediated apoptosis inducible by NKT cells. These results indicate that T and NKT differ functionally in their interaction with VSMCs.

**CD4+ T Cells Induce VSMC Proliferation**

**Whereas NKT Cells Induce VSMC Apoptosis**

Next, we investigated the effect of positively selected CD4+ T or NKT cells on VSMC proliferation by direct cell-cell contact. Fixed CD4+ T cells enhanced autologous VSMC proliferation on contact (Figure 6a). The mechanism involved is currently not clear as the proliferation is not affected by blocking VCAM-1, ICAM-1, or CD40 ligation with T cells that were pretreated with anti–VLA-4, anti–LFA-1, and anti-CD40L antibody. Interestingly, this process was contrasted by our demonstration in this study that NKT cells induced autologous VSMCs to apoptosis. This was demonstrated by the inhibition of VSMC proliferation in the presence of fixed NKT cells (Figure 6b). The inhibition was almost completely reversed by the presence of the broad-spectrum caspase inhibitor z-VAD-FMK, suggesting that NKT cells were inducing VSMC apoptosis. The induction of apoptosis in VSMCs was further confirmed by staining VSMCs, treated or not with fixed NKT cells, with annexin V-FITC and propidium iodide. In the presence z-YVAD-FMK, the inhibitor specific for caspase 1/ICE and caspase 4, the reversal (40%) of apoptosis was, however, less dramatic (Figure 6c). This suggests that some other caspase(s) may also be involved in the apoptotic process. In addition, when VSMCs were cultured in the presence of IFN-γ or spent NKT culture medium (SN) containing 788pg/mL IFN-γ, they showed a substantial increase (50%) in expression of Fas compared with in the presence of medium (med) alone (Figure 6d), indicating that IFN-γ produced by NKT1 cells could upregulate Fas expression on VSMCs. This could synergistically promote FasL-mediated apoptosis inducible by NKT cells. These results indicate that T and NKT differ functionally in their interaction with VSMCs.

**Discussion**

In this study, we show for the first time that apart from T cells, plaque-immune infiltrates in all 15 atherosclerotic AAA tissues examined contain a significant proportion of NK and
αβTCR^+CD161^+ NKT cells, and this varies widely between individuals and may depend on the disease activity, consistent with the marked heterogeneity of AAA in general. Normal aorta does not contain inflammatory infiltrates and contains few immune cells.34,35 Because we are not studying the tissue distribution and density of these inflammatory cells with disease progression, a normal aorta was not used. The Th, NK, and NKT cells are strongly positive for the IL-18Rα chain and therefore are predominantly type 1 lymphoid cells that produce IFN-γ. Among our expanded lymphoid cells, up

Figure 4. Multicolor flow cytometric analysis of in vitro expanded NKT1 and Th1 cells derived from atherosclerotic plaques. a, Among the expanded innate and adaptive lymphocytes analyzed, CD4 is expressed by both the CD161^+αβTCR^+ NKT cells (R2, top middle) and the CD161^-αβTCR^- T cells (R3, top right). b, None of the CD4^-CD161^- NKT cells (R2) express TCRγδ (top right). c, In vitro, expanded and positively selected NKT cells are CD3^-CD161^- and these same cells (from region R2) are IL-18R^-ST2L^- (top right). d, On analysis, similarly expanded and sorted T cells are all CD3^-CD161^- and these cells (from R2) are also predominantly IL-18R^- (top middle). Lower level of IL-18R expression is due to downregulation of its expression by extensive culture of T cells in medium containing IL-2.29 To check the efficiency of the sorting process, some of these T cells were also stained for expression of CD4 and CD8 and they (from R2) are predominantly CD4^- T helper cells (top right). Numerical values in the dot plots denote percentage of cells within gated quadrants. This is representative of the T and NKT cells sorted for use in Figures 5 and 6.
to 65% of αβ TCR+CD161+ NKT cells express CD4 and no CD4+CD161+ NKT cells are γδ TCR positive. Because of this, we used the CD4+CD161+αβ TCR+ NKT cell subset to focus our study of the interaction of NKT cells with VSMCs. Hence, when plaque-derived NKT, T, and VSMCs are expanded in vitro and cocultured, CD4+ Th cells induce VSMC proliferation. VSMCs induce NKT but not CD4+ or CD8+ T cell proliferation. NKT but not CD4+ Th cells induce VSMC apoptosis. These results suggest a mechanism by which CD4+ Th and NKT cells may regulate VSMC proliferation and death, respectively, a pathological hallmark of atherosclerosis. This could have significant in vivo implications because apoptosis of VSMCs has recently been identified as a key feature in plaque stability in atherosclerosis and is a major determinant of VSMC number in physiological remodeling of the vasculature.36

In mice, NKT cells have suppressor functions important in immunoregulation and prevention of autoimmunity in mice.37 However, unlike other T-regulatory cells, they can also act as aggressors in immune responses to some pathogens37 and as suppressors in anti-tumor immunity.22 This dual function of NKT cells is attributed to their ability to rapidly secrete cytokines associated with type 1 (IFN-γ) or type 2 (IL-4) responses on TCR engagement and reaction with glycolipid antigen.19,23 Cytokines such as IL-12 and IL-18 can also stimulate NKT cells to release IFN-γ and exhibit natural cytotoxicity.38 Furthermore, on activation, NKT cells also exert potent cytolytic activity mediated by perforin/granzyme B and FasL.22,24 In this study, we present data to show that NKT cells may be immunosuppressors in atherosclerosis. This is supported by our demonstration that NKT cells can inhibit VSMC growth by inducing them to apoptose via Fas/FasL interaction, a process that is mediated by ICE-like proteases/caspases28,60 and so is almost completely reversed by the broad spectrum caspase inhibitor z-VAD-FMK (Figure 6b). Furthermore, we show that IFN-γ produced by type 1 NKT (NKT1) cells in culture, can upregulate Fas expression on VSMCs (Figure 6d). This may synergistically promote the Fas/FasL-mediated VSMC apoptosis induced by NKT cells. As Fas/FasL-mediated apoptosis may be triggered in VSMCs at sites of high inflammatory content,36 our results suggest that NKT1 cells in plaque infiltrates may produce IFN-γ to sensitize VSMCs and induce apoptosis by Fas/FasL interaction. This could lead to plaque rupture when NKT1 cells are present in the shoulder region of plaques. In addition, NKT cells present in the media could also contribute to extracellular matrix degradation and pronounced decrease in medial VSMC density common in advanced AAA,41 as a result of VSMC apoptosis.

We have also demonstrated for the first time that there is strong CD40/CD40L interaction between NKT and VSMCs (Figure 5a), showing that VSMCs can induce NKT cell proliferation via CD40/CD40L interaction. In the context that NKT cells in turn can induce VSMC apoptosis, this may act as a negative feedback mechanism to reduce exuberant VSMC proliferation, or this may be a property of the VSMCs that have been isolated from the particular microenvironment of the aneurysm, where loss of VSMCs and hence vessel wall weakness is a key part of the pathophysiology. We also note that CD40 ligation activates Caspase1/ICE activity as well as the production of matrix-degrading enzymes in VSMCs,42,43 both of which can lead to apoptosis.36 Although NKT1 can induce apoptosis in VSMCs via Fas/FasL interaction, other stimuli and pathways exist that may act synergistically with Fas. This is demonstrated in Figure 6c where z-YVAD-FMK, a selective inhibitor of Caspase 1 can only partially reverse (40%) the VSMC apoptosis induced via CD40 ligation by fixed NKT1, leaving the remaining concurrent Fas-mediated apoptosis, where caspase 8 is an essential and apical initiator of the Fas signaling pathway.44,45 and other effector caspases unaffected. Although caspase-1 is involved in Fas-mediated apoptosis of thymocytes, it does not play a requisite role.46 Therefore, the partial inhibitory effect of z-YVAD-FMK on ICE may reflect synergistic effect of the Fas and CD40 signaling pathways in NKT-induced VSMC apoptosis. Furthermore, NKT1 can also kill via granzyme B/perforin-mediated pathway23 although that pathway is not included in the present study using fixed NKT cells.

By contrast, CD4+ Th1 cells may enhance plaque stability by inducing VSMC proliferation (Figure 6a) through unknown mechanisms. Therefore, we show here for the first
time the different and opposing mechanistic roles of the innate (NKT) and adaptive (CD4⁺ T) lymphocytes isolated from atherosclerotic tissues. Because of their opposing functions, their balance in the plaque may determine its stability or susceptibility to rupture. In this context, it is not surprising that whereas Th1 cells also produce IFN-γ, although much less to sensitize Fas and possibly lower FasL expression, the net effect is insufficient to induce significant apoptosis in VSMCs.

In conclusion, our in vitro analysis of the interaction between VSMCs and Th1 or NKT1 cells within atherosclerotic tissue reveals further complexity in atherosclerosis. We demonstrate for the first time, not only the presence of type 1 NKT cells in plaques, in addition, we propose a mechanism by which they and CD4⁺ Th1 cells may regulate VSMC proliferation or apoptosis. CD4⁺ Th1 lymphocytes, via their ability to enhance VSMC proliferation, may help to maintain plaque stability through the formation of a firm fibrous cap. This may be followed by enhanced expansion of NKT1 cells produced by the increased presence of VSMCs. NKT1 cells, in turn via Fas, IFN-γ production, and CD40 signaling, may induce VSMC apoptosis. With time, a disproportionate accumulation of NKT1 cells in conjunction with appropriate costimulation may culminate in plaque rupture as a result of VSMC apoptosis induced by NKT1 cells. The increased presence and destabilizing role of NKT cells in plaques may also provide an explanation for the clinical observation that by lowering lipid levels, statins reduce acute adverse coronary events, and this correlates with diminished vascular inflammation and activation as it has been shown that glycolipids such as the synthetic glycolipid α-galactosylceramide are required for NKT cell stimulation and atherosclerotic lesions are known to have higher concentrations of glycolipids than unaffected aortas. The balance of acquired (T cells) and innate (NKT cells) immunity may be important in atherosclerotic disease regulation. This may be determined by the extent of CD40L signaling, required for the induction of NKT cell proliferation by VSMCs (Figure 5a), and the effect may explain the reduction...
of atherosclerosis seen on administration of neutralizing anti-CD40L antibody in mice. This study has not only provided us with new insights into the pathogenesis of lesion progression, but could lead to novel therapeutic approaches involving immune modulation. Specifically, through understanding the proatherogenic role of NKT cells, it could provide a better understanding of how to manipulate plaque stability and further reduce the acute thrombotic complications of this disease.

Acknowledgments

This work was supported by the British Heart Foundation and The Wellcome Trust.

References


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Circ Res. 2005;96:675-683; originally published online February 24, 2005;
doi: 10.1161/01.RES.0000160543.84254.f1
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Materials and Materials

Isolation of lymphoid cells and VSMC from plaque tissue

Each tissue specimen was cleared of peripheral blood and repeatedly rinsed with Hanks balanced salt solution (HBSS). The tissue was then minced into fine pieces and digested at 37 °C with collagenase type I (Worthington Biochemical Corp., USA) in HBSS. This was followed by digestion with trypsin-EDTA at 37 °C, washed with Iscove’s Modified Dulbecco’s Medium (IMDM) and the cell suspension collected and used for flow cytometry and culture of lymphoid cells. The remaining tissue was then transferred to tissue culture flasks containing Medium 199/IMDM supplemented with 15% FCS for isolation of human VSMCs by explant outgrowth.

Culture, in vitro expansion and positive selection of plaque derived lymphoid cells

The lymphoid cell suspension obtained from each plaque tissue digest, together with some contaminating VSMCs, was washed and resuspended in culture medium (CM) made up of IMDM medium supplemented with 15% pooled male human sera, penicillin/streptomycin, pyruvate, 2-mercaptoethanol, IL-2 and IL-15. Lymphoid cells were stimulated with immobilized anti-CD3 (mAb clone OKT3) in culture medium containing anti-CD28 (mAb clone 9.3) and anti-CD161 (mAb clone 191B8) as co-stimulators for T and NKT cells respectively1,2 in a CO2 incubator at 37 °C. The stimulated lymphoid cells were fed at regular intervals with CM and split when required.

In contrast to mouse NKT, ligation of CD161 in the absence of TCR stimulation does not result in human NKT cell activation2. Therefore, for co-culture experiments to examine VSMC-lymphoid cell interactions, the in vitro expanded lymphoid cells were positively selected for NKT or T cells using anti-CD4, anti-CD161 and anti-CD56 but without anti-CD3
or anti-TCRαβ, to avoid activation of the cells. The lymphoid cells were first screened by flow cytometry to estimate the proportion of CD4+CD56+/CD161+αβTCR+ NKT cells or CD4+CD56+/161+γδTCR+ T cells in culture before initial positive selection with anti-human CD4 magnetic beads (Dynal, UK). Since no CD4+CD56+/CD161+γδTCR+ T cells were detected (Fig 4b), depletion of γδT cells was considered unnecessary. CD4+ bead bound cells were detached from the beads and then incubated with anti-CD161 and anti-CD56 antibody (ImmunoKontact, UK). The cells were washed before incubation with sheep anti-mouse IgG magnetic beads (Dynal). Unbound cells (CD4+CD161–CD56– T cells) were collected while bead bound cells (CD4+CD56+CD161+ NKT cells) were rosetted. After incubation in culture medium for 6h, the NKT cells were physically detached from the beads by pipetting. The sorted T and NKT cells were confirmed by flow cytometry before use, in co-culture experiments with VSMCs.

Flow cytometry

We have previously shown that IL-18R and ST2L are stable cell surface markers specific for type 1 and type 2 lymphoid cells respectively.3-5 Therefore, to estimate the proportion of lymphoid cell subsets (T, NK and NKT) that may be type 1 or 2 cells, lymphoid cells isolated from plaque tissue were characterized ex-vivo by 3-colour FACS analysis in Fig. 2 as previously described 5. Briefly, the cells were stained with anti-human IL-18Rα (R&D Systems, UK) or anti-human ST2L (Morwell Diagnostics, Switzerland) and detected with biotinylated goat F(ab)2 fragment anti-mouse IgG (Molecular Probes, USA) and Streptavidin Per-CP (BD Biosciences, UK). This was followed by incubation with FITC conjugated anti-CD3 (Dako, UK), anti-human TCRαβ (BD Biosciences) or anti-Vα24 (Beckman Coulter, UK) and Phycoerythrin (PE) conjugated anti-CD4, anti-CD56 (BD Biosciences) or anti-CD161 (Beckman Coulter). Isotype controls were included to set gates.
To estimate the proportion of CD4⁺CD161⁺αβTCR⁺ NKT among *in vitro* expanded lymphoid cells in Fig.4a and 4b, anti-human CD4 (ARP 318, CFAR, National Institute for Biological Standards and Control, UK) was first added and detected with Streptavidin Per-CP as above. This was followed by incubation with FITC conjugated anti-human TCRαβ and PE conjugated anti-CD161 (Beckman Coulter). To confirm that CD4⁺CD161⁺ NKT cells do not express γδTCR, the lymphoid cells were stained with anti-human γδTCR (BD Biosciences) and detected with Streptavidin Per-CP as above. This was followed by incubation with FITC conjugated anti-CD4 (DAKO) and PE-conjugated anti-CD161. Isotype controls were included to set gates.

For FACS analysis of Type 1 or Type 2 T and NKT cells sorted from *in vitro* expanded lymphoid cultures in Fig 4c and 4d, anti-CD161 was added and detected with streptavidin Per-CP as above. This was followed by incubation with Allophycocyanin (APC)-conjugated anti-CD3 (DAKO), PE-conjugated anti-hIL18Rα (R&D Systems) and FITC-conjugated anti-ST2L (Morwell Diagnostics) for both NKT and T cells. To check for purity of CD4⁺ T cells from positive selection, some T cells were also stained, after the CD161/streptavidin-PcP step, with APC-conjugated anti-CD3, FITC-conjugated anti-CD4 (DAKO) and PE-conjugated anti-CD8 (BD Pharmingen). Isotype controls were included to set gates. Expression of cell surface antigens was analysed on a BD FACS Calibur flow cytometer. All flow cytometric analysis was conducted with WinMDI version 2.8 and the numerical values in the dot plots denote percentage of cells within gated quadrants.

**Histology**

For histological analysis (Fig 1a) of the distribution of inflammatory cell infiltrate, elastin, collagen and lipid content in human tissues of atherosclerotic aneurysm, consecutive cryostat sections (5µm) were stained with haematoxylin and eosin (HE, top left panel), Oil red
O with haematoxylin (OROH, top right panel) or Verhoeff von Giesson (VG, bottom panel). For HE staining, the tissue was stained in Gills haematoxylin, washed in water, counterstained in Eosin, dehydrated, cleared in xylene and mounted. To stain for lipids, the tissue was placed in 0.5% Oil red O solution and counterstained in Gills haematoxylin before examination. The distribution of elastin and collagen were monitored by staining the tissue in Verhoeff’s solution, differentiating in 2% aqueous ferric chloride, then in 5% sodium thiosulphate to remove excess iodine and counterstained in Von Giesson’s solution before observation. The images were captured with a light microscope (E800 Nikon, Japan) equipped with a digital camera.

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For immunohistochemical analyses, cryostat sections (5 µm) consecutive to those used for histological analysis were used (Fig 1b). For triple immuno-visualization, a method of simultaneous detection of tissue antigens was applied as previously described. Briefly, tissue sections were first incubated with rabbit anti-human IL-18R antibody followed by incubation with alkaline phosphatase conjugated goat anti-rabbit IgG (Dako, UK). The reaction was visualized by adding substrate solution (Alkaline Phosphatase Vector blue substrate kit, Vector Labs.) containing levamisole solution to block endogenous alkaline phosphatase. Tissue sections were then incubated with mAb anti-human αβ TCR (Beckman Coulter) followed by incubation with Alexa 488-conjugated goat F(ab)_2 fragment anti-mouse IgG2b (Molecular Probes). Tissues were then blocked with Avidin/biotin block (Vector Labs.) before biotinylated monoclonal anti-human CD161 antibody (Serotec, UK) was next added followed by incubation with Alexa 546-conjugated streptavidin (Molecular Probes). Isotype control normal rabbit and mouse IgG were used as negative control for primary antibodies. Tissue sections were washed with PBS between all incubations and normal goat IgG was used
to block non-specific binding. The sections were observed under a fluorescence microscope (E800 Nikon, Japan) and images captured with a Zeiss confocal laser scanning microscope LSM510 equipped with a digital camera.

For immunocytochemical analyses, VSMC were grown on poly-L-lysine coated coverslips for 24 h before lymphoid cells were added (Fig 3). The cells were fixed with 1% paraformaldehyde followed by cell membrane permeabilization with cold absolute ethanol. All staining procedures were basically as above except that the antibodies used were anti-CD3 antibody detected with biotinylated goat F(ab)_2 fragment anti-mouse IgG and Streptavidin-AP, anti-human CD161 antibody detected with Alexa 546-conjugated goat anti-mouse IgG, and FITC-conjugated anti-human smooth muscle cell actin (Sigma, UK). Isotype control normal mouse IgG was used throughout as negative control for primary antibodies. Images were captured with a fluorescence microscope (E800 Nikon, Japan) equipped with a digital camera.

**NKT or Th cell proliferation in the presence of fixed VSMC**

VSMC (8X10^3/well) were cultured for 24 h before incubation with anti-VCAM-1 (ImmunoKontact, UK), anti-CD40 (Chemicon, UK), anti-ICAM-1 (Beckman Coulter, UK) or isotype control antibody, all at 40 µg/ml, washed thrice with medium and then fixed at +4 °C with 1% paraformaldehyde. The fixed VSMCs were washed with medium before CD4^+CD161^+ NKT or CD4^+ Th cells in CM medium were added at a ratio of between 5-10 lymphoid cells: 1 VSMC. The lymphoid cells (in triplicates) were then incubated for 24 h at 37 °C and pulsed during the last 12 h with ^3^H-thymidine. Incorporated radioactivity was determined with a scintillation counter.
**VSMC proliferation in the presence of fixed Th or NKT cells**

CD4⁺ Th cells were pre-incubated with anti-CD40L, anti-VLA-4 and anti-LFA-1 antibodies (all from Beckman Coulter) or isotype control antibody, all at 40 µg/ml, washed and fixed at +4 °C with 1% paraformaldehyde. They were then washed thrice and resuspended in CM. CD4⁺CD161⁺ NKT cells were similarly fixed but without pretreatment with antibodies. 100 µl of the fixed pretreated or not Th, NKT or medium control were then added to freshly trypsinized VSMC (4X10³/well) in 100 µl M199 + 10% FCS within 5 h of VSMC plating, at a ratio of 10 Th or NKT cells: 1 VSMC. For VSMC cultures with fixed NKT, the broad-spectrum caspase inhibitor z-VAD-FMK (ICN, UK) was added to VSMC at 20 µM about 10 min before adding fixed NKT cells. The whole, in triplicates, was incubated for 10 h and then pulsed with ³H-thymidine for 27 h before harvesting. Incorporated radioactivity was determined with a scintillation counter.

**Detection of apoptosis in co-cultures of VSMC and fixed NKT**

For apoptosis determination, 2X10⁴ VSMC/well were used and the cells were cultured for 40 h after addition of fixed NKT or medium (control), before they were harvested and stained with annexin V-FITC and propidium iodide (Pharmingen, USA) according to the manufacturers instructions and analysed by flow cytometry. The caspase 1/ IL-1β-converting enzyme (ICE) inhibitor z-YVAD-FMK (Calbiochem, UK) was added to VSMC at 20µM about 10 min before adding fixed NKT cells.

**FACS analysis of Fas expression by VSMC**

Spent NKT culture supernatant (SN), recombinant IFNγ (5000 U) or medium was added to an overnight culture of VSMC (2 X 10⁴/well) and the cells were further incubated for 24 h at 37 °C in a CO₂ incubator. The VSMC were gently washed and harvested with
trypsin/EDTA. To ensure that Fas is expressed by VSMCs, 2-colour flow cytometry was carried out to stain Fas on the cell surface and intracellular \( \alpha \)-actin fibres in VSMCs. Therefore the cells were first incubated with chilled ethanol to permeabilise their cell membrane for antibody penetration. Then they were incubated with anti-Fas (Beckman Coulter) or isotype control IgG (MOPC-31) and visualized with PE-conjugated Rabbit F(ab)\(_2\) fragment anti mouse IgG (DAKO). This was followed by incubation with FITC-conjugated anti-\( \alpha \)-smooth muscle actin (SIGMA) or isotype control antibody. Expression of Fas on \( \alpha \)-actin\(^+\) VSMCs was analysed by flow cytometry.

**IL-15 secretion by VSMC**

IL-15 that was secreted by VSMC cultured at 1X10\(^6\) cells/ml of medium with or without 5000 u/ml recombinant IFN\( \gamma \) for 48 h was measured by ELISA assay in triplicates. Recombinant hIL-15 (R & D Systems, USA) was used as standard.

**Statistical Analysis**

Results are expressed as mean \( \pm \) SEM. Statistical analysis was carried out with SigmaStat (SPSS) software. Student’s \( t \) test was used for statistical analysis between two experimental groups after testing that the data complied with the constraints of parametric analysis (Figures. 5b and 5c). For data from multiple groups (Figures 5a, 6a and 6b), one-way analysis of variance ANOVA was used to compare each group. Post hoc Dunnett’s test for comparison of groups with control condition were then performed to identify which group differences account for the significant overall ANOVA. Where parametric analysis was not permissible, analysis between matched pairs were conducted using Wilcoxon Signed Ranks test (Figure. 2). Values with \( P < 0.05 \) were considered statistically significant.
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Table 1  Baseline characteristics of patients ( n = 15 )

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* Values are percent of total number of patients studied. ** Below normal range. The risk factors for atherosclerosis in the study group include hypertension, smoking, age and male gender.
**Materials and Materials**

**Isolation of lymphoid cells and VSMC from plaque tissue**

Each tissue specimen was cleared of peripheral blood and repeatedly rinsed with Hanks balanced salt solution (HBSS). The tissue was then minced into fine pieces and digested at 37 °C with collagenase type I (Worthington Biochemical Corp., USA) in HBSS. This was followed by digestion with trypsin-EDTA at 37 °C, washed with Iscove’s Modified Dulbecco’s Medium (IMDM) and the cell suspension collected and used for flow cytometry and culture of lymphoid cells. The remaining tissue was then transferred to tissue culture flasks containing Medium 199/IMDM supplemented with 15% FCS for isolation of human VSMCs by explant outgrowth.

**Culture, in vitro expansion and positive selection of plaque derived lymphoid cells**

The lymphoid cell suspension obtained from each plaque tissue digest, together with some contaminating VSMCs, was washed and resuspended in culture medium (CM) made up of IMDM medium supplemented with 15% pooled male human sera, penicillin/streptomycin, pyruvate, 2-mercaptoethanol, IL-2 and IL-15. Lymphoid cells were stimulated with immobilized anti-CD3 (mAb clone OKT3) in culture medium containing anti-CD28 (mAb clone 9.3) and anti-CD161 (mAb clone 191B8) as co-stimulators for T and NKT cells respectively1,2 in a CO2 incubator at 37 °C. The stimulated lymphoid cells were fed at regular intervals with CM and split when required.

In contrast to mouse NKT, ligation of CD161 in the absence of TCR stimulation does not result in human NKT cell activation2. Therefore, for co-culture experiments to examine VSMC-lymphoid cell interactions, the in vitro expanded lymphoid cells were positively selected for NKT or T cells using anti-CD4, anti-CD161 and anti-CD56 but without anti-CD3
or anti-TCRαβ, to avoid activation of the cells. The lymphoid cells were first screened by flow cytometry to estimate the proportion of CD4⁺CD56⁺/CD161⁺αβ TCR⁺ NKT cells or CD4⁺CD56⁺/CD161⁺γδ TCR⁺ T cells in culture before initial positive selection with anti-human CD4 magnetic beads (Dynal, UK). Since no CD4⁺CD56⁺/CD161⁺γδ TCR⁺ T cells were detected (Fig 4b), depletion of γδ⁺ T cells was considered unnecessary. CD4⁺ bead bound cells were detached from the beads and then incubated with anti-CD161 and anti-CD56 antibody (ImmunoKontact, UK). The cells were washed before incubation with sheep anti-mouse IgG magnetic beads (Dynal). Unbound cells (CD4⁺CD161⁻CD56⁻ T cells) were collected while bead bound cells (CD4⁺CD56⁺CD161⁺ NKT cells) were rosetted. After incubation in culture medium for 6h, the NKT cells were physically detached from the beads by pipetting. The sorted T and NKT cells were confirmed by flow cytometry before use, in co-culture experiments with VSMCs.

**Flow cytometry**

We have previously shown that IL-18R and ST2L are stable cell surface markers specific for type 1 and type 2 lymphoid cells respectively.³⁻⁵ Therefore, to estimate the proportion of lymphoid cell subsets (T, NK and NKT) that may be type 1 or 2 cells, lymphoid cells isolated from plaque tissue were characterized ex-vivo by 3-colour FACS analysis in Fig. 2 as previously described.⁵ Briefly, the cells were stained with anti-human IL-18Rα (R&D Systems, UK) or anti-human ST2L (Morwell Diagnostics, Switzerland) and detected with biotinylated goat F(ab)₂ fragment anti-mouse IgG (Molecular Probes, USA) and Streptavidin Per-CP (BD Biosciences, UK). This was followed by incubation with FITC conjugated anti-CD3 (Dako, UK), anti-human TCRαβ (BD Biosciences) or anti-Vα24 (Beckman Coulter, UK) and Phycoerythrin (PE) conjugated anti-CD4, anti-CD56 (BD Biosciences) or anti-CD161 (Beckman Coulter). Isotype controls were included to set gates.
To estimate the proportion of CD4⁺CD161⁺αβTCR⁺ NKT among in vitro expanded lymphoid cells in Fig.4a and 4b, anti-human CD4 (ARP 318, CFAR, National Institute for Biological Standards and Control, UK) was first added and detected with Streptavidin Per-CP as above. This was followed by incubation with FITC conjugated anti-human TCRαβ and PE conjugated anti-CD161 (Beckman Coulter). To confirm that CD4⁺CD161⁺ NKT cells do not express γδTCR, the lymphoid cells were stained with anti-human γδTCR (BD Biosciences) and detected with Streptavidin Per-CP as above. This was followed by incubation with FITC conjugated anti-CD4 (DAKO) and PE-conjugated anti-CD161. Isotype controls were included to set gates.

For FACS analysis of Type 1 or Type 2 T and NKT cells sorted from in vitro expanded lymphoid cultures in Fig 4c and 4d, anti-CD161 was added and detected with streptavidin Per-CP as above. This was followed by incubation with Allophycocyanin (APC)-conjugated anti-CD3 (DAKO), PE-conjugated anti-hIL18Rα (R&D Systems) and FITC-conjugated anti-ST2L (Morwell Diagnostics) for both NKT and T cells. To check for purity of CD4⁺ T cells from positive selection, some T cells were also stained, after the CD161/streptavidin-PcP step, with APC-conjugated anti-CD3, FITC-conjugated anti-CD4 (DAKO) and PE-conjugated anti-CD8 (BD Pharmingen). Isotype controls were included to set gates. Expression of cell surface antigens was analysed on a BD FACS Calibur flow cytometer. All flow cytometric analysis was conducted with WinMDI version 2.8 and the numerical values in the dot plots denote percentage of cells within gated quadrants.

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