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Increase of Vδ2+ T Cells That Robustly Produce IL-17A in Advanced Abdominal Aortic Aneurysm Tissues

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ABSTRACT

Abdominal aortic aneurysm (AAA) is a chronic dilation of the aorta with a tendency to enlarge and eventually rupture, which constitutes a major cause of cardiovascular mortality. Although T-cell infiltrates have been observed in AAA, the cellular, phenotypic, and functional characteristics of these tissue-infiltrating T cells are not fully understood. Here, we investigated the proportional changes of T-cell subsets—including CD4+ T cells, CD8+ T cells, and γδ T cells—and their effector functions in AAAs. We found that Vδ2+ T cells were presented at a higher frequency in aortic aneurysmal tissue compared to normal aortic tissue and PBMCs from patients with AAA. In contrast, no differences were observed in the frequencies of CD4+, CD8+, and Vδ1+ T cells. Moreover, we observed that the Vδ2+ T cells from AAA tissue displayed immunophenotypes indicative of CCR5+ non-exhausted effector memory cells, with a decreased proportion of CD16+ cells. Finally, we found that these Vδ2+ T cells were the main source of IL-17A in abdominal aortic aneurysmal tissue. In conclusion, our results suggest that increased Vδ2+ T cells that robustly produce IL-17A in aortic aneurysmal tissue may contribute to AAA pathogenesis and progression.

Keywords: Abdominal aortic aneurysm; Gamma Delta T cells; IL-17A

INTRODUCTION

Abdominal aortic aneurysm (AAA) is a progressive segmental abdominal aortic dilation that is associated with high mortality. AAAs account for up to 15,000 deaths per year, mainly due to aortic aneurysm rupture (1,2), constituting the 13th most common cause of death in the United States (3). Unfortunately, there is no presently available pharmacological agent that reliably limits AAA expansion, and thus surgical or endovascular interventions are recommended to prevent the rupture of large AAAs (4). Elucidating the molecular mechanism underlying aortic aneurysm is highly important to guide the development of new therapeutic modalities.

The putative mechanisms involved in aortic aneurysm development and progression are quite complex. It has been suggested that disease progression may be associated with innate and adaptive immunity (5), atherosclerosis, reactive oxygen species, and extracellular matrix degradation (6). Aortic aneurism tissue exhibits a predominance of T lymphocytes, along with B lymphocytes, macrophages, mast cells, and natural killer cells (4,7), implying that adaptive immunity plays a crucial role in AAA pathogenesis. Indeed, evidence supports that adaptive immune responses are involved in vascular inflammation, and the consequent development of atherosclerosis and aortic aneurysm (4,7,8).

Among various T-lymphocyte subsets, γδ T cells constitute a small proportion of total immune cells, and are usually detected in secondary lymphoid tissues, such as gastrointestinal lymph nodes and intestinal mucosa-associated follicles (9). However, this cell population is closely related to adaptive immune response regulation through antigen presentation and immune modulation (10). A prior study of human AAA described γδ T-cell infiltration and clonal expansion in aortic tissue (11). Although experimental studies suggest that this cell subset plays a putative role in the pathogenesis of atherosclerosis and aneurysm, direct evidence of its involvement in human aortic aneurysm is scarce.

In this study, we used multicolor flow cytometry to characterize the proportional changes of T-cell subsets—e.g., CD4+ T cells, CD8+ T cells and γδ T cells—in AAA tissue compared with in normal aortic tissue and PBMCs. We additionally performed *in vitro* experiments to examine the production of pro-inflammatory cytokines and cytotoxic molecules by CD4+ T cells, CD8+ T cells, and γδ T cells.

MATERIALS AND METHODS

Patients and specimens
This study included nine patients who were diagnosed with AAA and hospitalized at Severance Hospital (Seoul, Korea). All patients underwent graft replacement surgery. From each patient, fresh aortic tissue, whole blood, and serum samples were collected. **Table 1** presents information about the nine AAA patients. This study was conducted in accordance with the principles of the Declaration of Helsinki, and approved by the Institutional Review Boards of Severance Hospital (approval No. 4-2019-1047). Each patient gave their written informed consent prior to inclusion in the study.

Lymphocyte isolation
PBMCs were separated by standard Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation, and then cryopreserved in FBS with 10% DMSO (Sigma-Aldrich,

Table 1. Clinical characteristics of the study patients

Patients	Sex	Age	Type	Aortic diameter (cm)	Rupture	Diabetes mellitus	Hypertension
1	M	79	AAA	4.5	No	No	Yes
2	M	78	AAA	6.4	No	No	No
3	M	76	AAA	6.0	No	No	Yes
4	M	64	AAA	11.0	No	No	Yes
5	M	84	AAA	8.7	No	No	Yes
6	M	78	AAA	5.5	No	No	Yes
7	M	76	AAA	9.5	No	Yes	Yes
8	M	71	AAA	7.6	No	No	Yes
9	M	64	AAA	6.8	No	Yes	Yes

St. Louis, MO, USA) until use. To purify single-cell suspensions of lymphocytes from tissue samples, aortic tissue was placed in a gentle MACS C-Tube (Miltenyi Biotec, Bergisch Gladbach, Germany) enzyme H, enzyme R, and enzyme A (Miltenyi Biotec) pre-mixed in RPMI. These C-tubes were then placed on a gentle MACS Octo Dissociator. Next, the cell suspension was passed through a 70-μm cell strainer, and the cells were washed once and then resuspended in media. The number and viability of mononuclear cells assessed using a Cellometer® Auto 2000 (Nexcelom, Lawrence, MA, USA). The cells were then washed once and cryopreserved. CD45⁺ cells from aortic tissue were purified from single-cell isolates using the REALease CD45 (TIL) MicroBead Kit (130-121-563; Miltenyi Biotec).

Flow cytometry

Cryopreserved PBMCs and tissues were thawed, and then stained with fluorochrome-conjugated antibodies for 30 min at 4°C. To gate out dead cells, the PBMCs and aortic tissues were stained using the LIVE/DEAD Fixable Red or Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA). The cells were washed once, stained with fluorochrome-conjugated antibodies against surface markers for 30 min at 4°C, and then washed again. For intracellular staining, surface-stained cells were permeabilized using a Foxp3 staining buffer kit (eBioscience, Carlsbad, CA, USA), and then further stained for intracellular markers, including IFN-γ, TNF-α, IL-17A, granzymes, and perforin. Multicolor flow cytometry was performed using an LSR II instrument (BD Biosciences, San Jose, CA, USA), and the data were analyzed using FlowJo software (Treestar, Ashland, OR, USA). We used the following conjugated mAbs: anti-CD14 (PE-eFluor610; 61D3; Invitrogen), anti-CD16 (BV605; 3G8; BD Biosciences), anti-CD19 (PE-eFluor610; HIB19; Invitrogen), anti-CD3 (Af700; UCHT1; BD Biosciences), anti-CD4 (AF700; RPA-T4; BD Biosciences), anti-CD4 (BV605; RPA-T4; BD Biosciences), anti-CD8 (BV650; RPA-T8; BD Biosciences), anti-CD8 (APC-Cy7; SK1; BD Biosciences), anti-CD45RA (APC-H7; HI100; BD Biosciences), anti-CCR5 (BV421; 2D7/CCR5; BD Biosciences), anti-CCR7 (PerCP-Cy5.5; G043H7; BioLegend, San Diego, CA, USA), anti-CXCR5 (PE; J252D4; BioLegend), anti-PD-1 (BV421; EH12.2H7; BioLegend), anti-TCR Vd1 (PE-Cy7; TS8.2; Iinvitrogen), anti-TCR Vd2 (PE-Cy7; B6; BioLegend), anti-IFN-γ (BV421; B27; BD Biosciences), anti-TNF-a (PE-Cy7; MAb11; BioLegend), anti-IL-17A (BV711; BL168; BioLegend), anti-Granzyme B (Af647; GB11; BD Biosciences), and anti-Perforin (PE; dG9; BioLegend).

In vitro stimulation

PBMCs and tissues from AAA patients were cultured for 6 h in the presence of anti-CD3 (1 μg/mL; Miltenyi Biotec). Next, the gated CD4⁺, CD8⁺, or Vδ2⁺ T cells were examined for expression of cytokines and cytotoxic molecules, including IFN-γ, TNF-α, IL-17A, granzymes, and perforin.

Statistical analysis

Statistical analyses were performed using Prism 7 (GraphPad Software, San Diego, CA, USA). All data are presented as the mean±standard deviation. The Mann-Whitney *U* test was used to compare 2 groups in each experiment, and the Wilcoxon matched-pairs signed-rank test to compare 2 paired groups in each experiment. A - value of <0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Vδ2+ T cells are increased in abdominal aortic aneurysmal tissue

To characterize the proportional changes of T-cell subsets in AAA tissue, we first compared the frequencies of CD4+, CD8+, Vδ1+, and Vδ2+ T cells among CD3+ lymphocytes between PBMCs and lymphocytes from aortic aneurysm tissue (**Fig. 1A**). The gating strategy for Vδ2+ T cells or Vδ1+ T cells was shown in **Supplementary Fig. 1A**. The proportions of CD4+, CD8+, and Vδ1+ T cells did not differ between aneurysm tissue and PBMCs. However, the proportion of Vδ2+ T cells was significantly greater in aortic aneurysmal tissue compared to PBMCs (**Fig. 1A**). In addition, the frequency of regulatory T cells in CD4+ T cells was significantly lower in aortic aneurysmal tissue compared to PBMCs (**Supplementary Fig. 1B**). When we then compared the proportions of Vδ1+ and Vδ2+ T cells between aortic aneurysmal tissue and normal aortic tissue, the proportion of Vδ2+ T cells, not Vδ1+ T cells, was significantly higher in aneurysmal tissue, compared to normal aortic tissues (**Fig. 1B and C**). These results suggested that aortic aneurysmal tissue exhibited a predominant increase of Vδ2+ T cells, without substantial increases of other T-cell populations, such as CD4+, CD8+, and Vδ1+ T cells.

Next, we examined the detailed characteristics of Vδ2+ T cells in aneurysmal aortic tissue. We found that aneurysmal aortic tissue did not include CCR7+CD45RA+ naïve Vδ2+ T cells, and exhibited minimal presence of CD45RA-expressing effector memory (CCR7-CD45RA+, EMRA) cells, while Vδ2+ T cells in PBMCs exhibited a significant portion of CCR7-CD45RA+ cells. On the other hand, CCR7-CD45RA- effector memory cells constituted the major subset of Vδ2+ T cells in aneurysmal aortic tissue (**Fig. 1D**). Similarly, effector memory cells were the predominant cell type observed among CD4+ and CD8+ T cells in aneurysmal aortic tissue (**Fig. 1D**).

We then performed flow cytometry to assess the expression of surface markers related to γδ T-cell effector functions, including CCR5, CXCR5, PD-1, and CD16. Since CCR5 and CXCR5 are known to act as homing markers of Vδ2+ T cells to tissue (12,13), we examined the expressions of these chemokine receptors on Vδ2+ T cells from aortic aneurysmal tissue (**Fig. 1E and F**). The frequency of CXCR5+ cells was significantly increased among Vδ2+ T cells from aortic aneurysmal tissue compared to normal aorta tissue and PBMCs from aneurysm patients (**Fig. 1F**). In contrast, the frequency of CCR5+ cells among Vδ2+ T cells did not significantly differ between aneurysmal aortic tissue, normal aortic tissue, and PBMCs from aneurysm patients (**Fig. 1E**). These findings suggest that upregulated-expression of CXCR5 and maintained-expression of CCR5 contribute to the migration of Vδ2+ T cells to aortic aneurysm tissue during AAA. We also measured expression of the T-cell exhaustion marker PD-1, and found that the percentage of PD-1+ Vδ2+ T cells did not differ between normal aortic tissue and aneurysmal aortic tissue (**Fig. 1G**). Previous studies have reported that antibody-dependent cellular cytotoxicity (ADCC) is a mechanism related to the effector functions of Vδ2+ T cells (14). However, we found that expression of the ADCC marker CD16 was significantly lower among Vδ2+ T cells from aortic aneurysmal tissue compared to normal aortic tissue and PBMCs (**Fig. 1H**). Taken together, these results indicated that Vδ2+ T cells are non-exhausted effector memory cells, and that their effector functions may not be mediated by direct killing activity against target cells.

Vδ2+ T cells in abdominal aortic aneurysmal tissue robustly produce IL-17A

It has been suggested that pro-inflammatory cytokine production by Vδ2+ T cells plays an important role in the induction of these cells' effector function. Previous reports show high-level production of IFN-γ and TNF-α by Vδ2+ T cells upon TCR stimulation (15,16). Moreover,
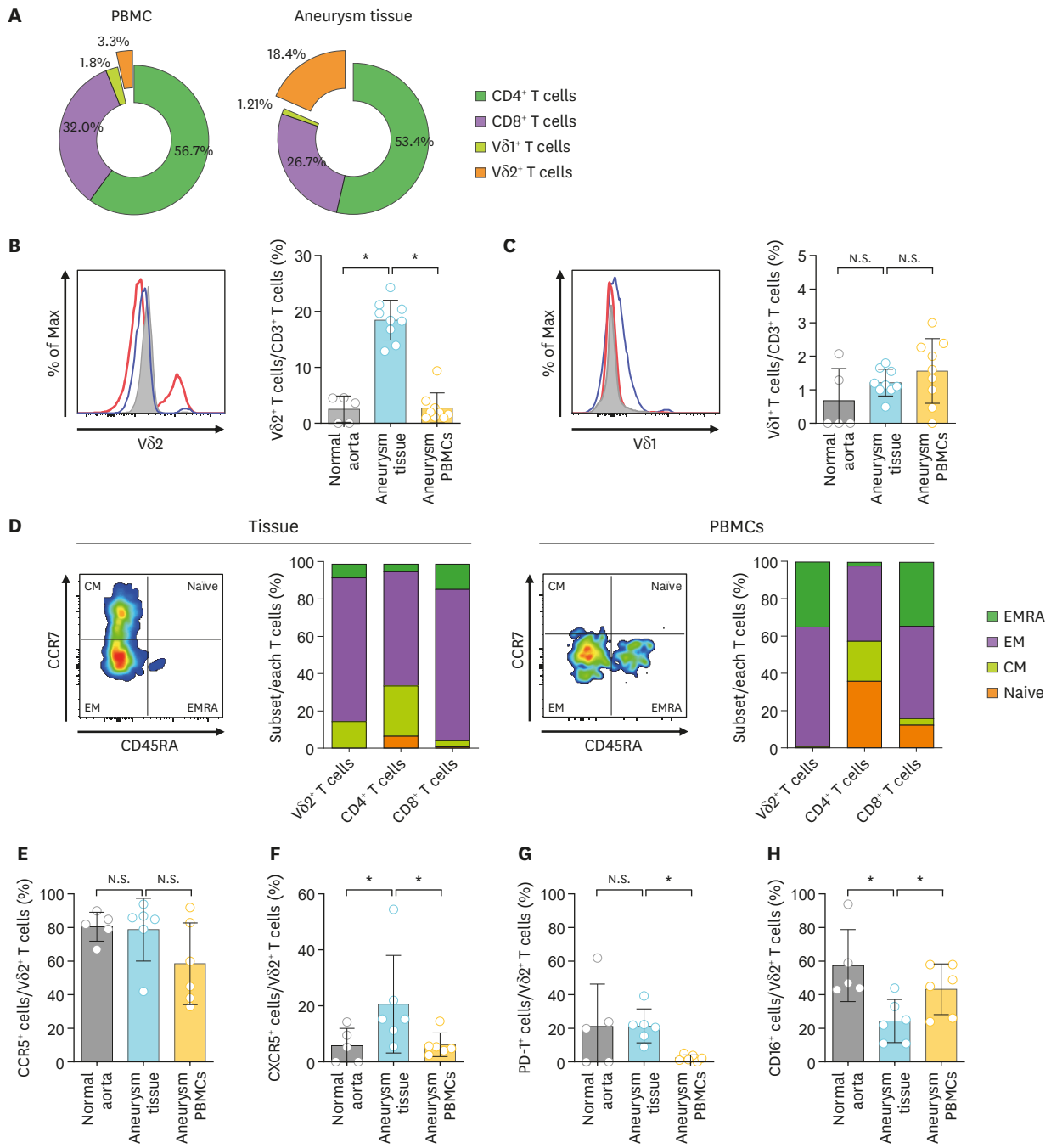
Figure 1. Increased proportion of Vδ2⁺ T cells in aortic aneurysmal tissue. (A) Flow cytometry analysis of the subset of CD3⁺ T cells in PBMCs and aortic tissue from AAA patients (n=9). Pie graph showing the proportions of subsets among CD3⁺ T cells from PBMCs and tissue (left panel). Percentages of Vδ2⁺ T cells among CD3⁺ T cells from PBMCs and tissue (right panel). (B, C) *Ex vivo* flow cytometry analysis of normal aortic tissue (n=5), aortic aneurysmal tissue (n=9), and PBMCs from AAA patients (n=9). The frequencies of Vδ2⁺ T cells and Vδ1⁺ T cells among CD3⁺ T cells were examined. A representative FACS plot (left panel). Summary data (right panel). (D) Flow cytometry analysis using the surface markers CD45RA and CCR7, of subsets of Vδ2⁺ T cells in aortic aneurysmal tissue from AAA patients (n=9). The frequency of Vδ1⁺ T cells among CD3⁺ T cells was examined. A representative FACS plot (left panel). Summary data (right panel). (E-H) *Ex vivo* flow cytometry analysis of number\Normal aortic tissue (n=5), aortic aneurysmal tissue (n=6), and PBMCs from AAA patients (n=6). The frequencies of CCR5⁺ cells (E), CXCR5⁺ cells (F), PD-1⁺ cells (G), and CD16⁺ cells (H) among Vδ2⁺ T cells were examined, and summary data are presented. Data are presented as mean and SD. Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test or Mann-Whitney *U* test.
N.S., not significant; EMRA, CD45RA-expressing effector memory; EM, effector memory; CM, central memory.
*p<0.05.

Th17-polarized Vδ2+ T cells produce IL-17A, which triggers proinflammatory cytokine and chemokine production by several immune cells ([17],[18]). Vδ2+ T cells also reportedly secrete cytotoxic molecules, such as granzyme and perforin, which contribute to the control of microbial infection ([19],[20]). However, the immunological profile of the effector molecules produced by Vδ2+ T cells in aneurysmal aortic tissue has not been elucidated. Therefore, we next examined the effector functions of Vδ2+ T cells in aneurysmal aortic tissue by measuring the *ex vivo* levels of pro-inflammatory cytokines without anti-CD3 stimulation, including IFN-γ, TNF-α, and IL-17A, as well as the cytotoxic molecules perforin and granzyme B.

Intriguingly, the frequency of IL-17A+ cells was significantly higher among Vδ2+ T cells compared to CD4+ and CD8+ T cells from aortic aneurysmal tissue (**Fig. 2A**). We confirmed the IL-17A+ cells from aortic aneurysmal tissue were predominantly Vδ2+ T cells among the CD3+ lymphocyte population (**Fig. 2B**). Furthermore, in AAA patients, we detected IL-17A-producing Vδ2+ T cells only in aortic tissue, not among PBMCs (**Fig. 2C**). We examined the frequencies of IFN-γ+ and TNF-α+ among CD4+, and CD8+ in aneurysmal tissue and the frequencies of IFN-γ+ and TNF-α+ Vδ2+ T cells in tissue and PBMCs. We found that aortic aneurysmal tissue and PBMCs exhibited very low frequencies of IFN-γ+ and TNF-α+ Vδ2+ T cells, which were similar to the frequencies among CD4+ and CD8+ T cells (**Fig. 2D and E**). Intriguingly, the percentages of granzyme B+ and perforin+ cells were significantly decreased among Vδ2+ T cells compared to among CD8+ T cells from aortic aneurysmal tissue (**Fig. 2F**), and the frequency of granzyme B+ cells among Vδ2+ T cells was significantly lower in aortic aneurysmal tissue compared to PBMCs (**Fig. 2G**). We observed similar results in Vδ2+ T cells upon stimulation with anti-CD3 to investigate remnant function (**Supplementary Fig. 2**). Collectively, our results demonstrated that the pro-inflammatory cytokine IL-17A was mainly produced by Vδ2+ T cells in aneurysmal tissue, implying that IL-17A-producing Vδ2+ T cells may play an important role in the progression of AAA.

In this study, we demonstrated the increased presence of Vδ2+ T cells in aortic aneurysmal tissues from patients with AAA. Among the various T-lymphocyte subsets located in the excised aneurysmal sac, the prevalence of Vδ2+ T cells was notably higher in aneurysm tissue compared to in peripheral blood or normal aorta tissue. Also, IL-17 expression was markedly increased in the Vδ2+ T cells from human aortic aneurysm tissue. Overall, these results imply that Vδ2+ T cells may be involved in AAA progression, and specifically appear to play an immuno-modulatory role on the aortic wall, rather than exerting direct cytotoxic activity. Vδ2+ T cell migration mediated by the changes of chemokine receptor expressions and a niche-induced proliferation might be a reason for increased Vδ2+ T cells in aortic aneurysm tissue, but further studies for understanding the detailed mechanisms are deeded. To our knowledge, this is the first report demonstrating the predominant presence of Vδ2+ T in advanced human AAA tissues, and the distinct immunological characteristics of this cell population.

Among the various T-lymphocyte subsets, γδ T cells constitute 1-5% of the circulating T cells in peripheral blood ([21]). However, the γδ T-cell subset can rapidly expand in response to phosphorylated metabolites released from external microorganisms or stressed cells ([22]). Upon activation, γδ T cells differentiate into effector memory cells and central memory cells, and retain distinct Th1-, Th2-, Th17- and T regulatory-like effector functions, thus exerting a regulatory role on the cell-mediated and humoral immune responses ([23]). Intriguingly, intracellular cholesterol contents regulate the activation, proliferation, and effector memory function of γδ T cells ([24]), implying a possible role in atherosclerotic lesions. However, further studies are needed to elucidate the distinct roles of this T-cell subset.
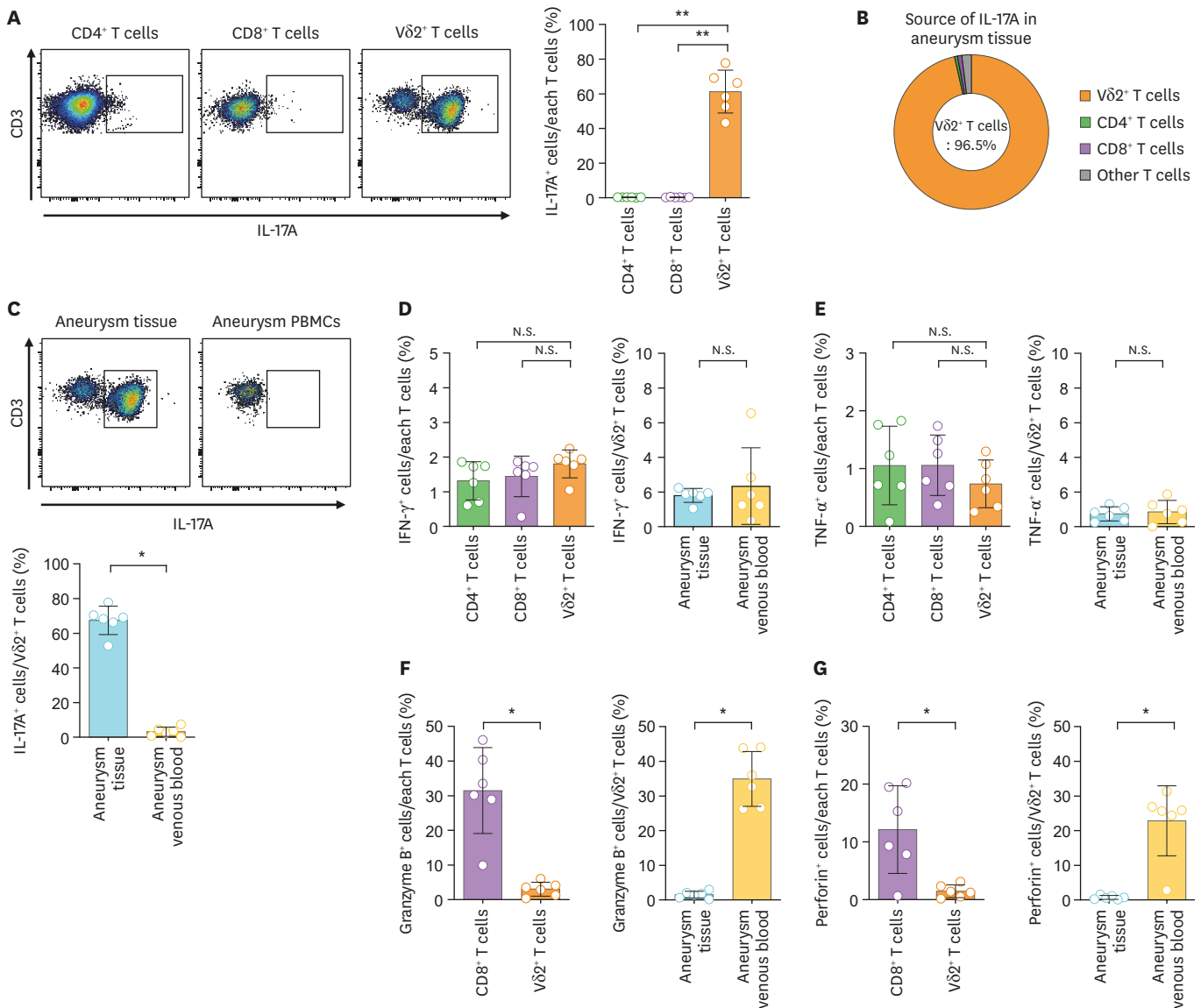
Figure 2. Vδ2⁺ T cells are the main source of IL-17A in aortic aneurysmal tissue. (A-G) IL-17A, IFN-γ, TNF-α, perforin and granzyme B were analyzed by flow cytometry in *ex vivo* without anti-CD3 stimulation. (A) *Ex vivo* flow cytometry analysis of IL-17A⁺ cells among CD4⁺, CD8⁺, and Vδ2⁺ T cells in aortic tissue from AAA patients (n=6). The frequency of IL-17A⁺ T cells in each T-cell subset was examined. A representative FACS plot (left panel). Summary data (right panel). (B) Flow cytometry analysis of the proportions of cell types among IL-17A⁺ CD3⁺ T cells in aortic aneurysmal tissue (n=6). Pie graph shows the proportions of cell subsets among IL-17A⁺ CD3⁺ T cells from tissue. (C) *Ex vivo* flow cytometry analysis of IL-17A⁺ cells among Vδ2⁺ T cells in aortic tissue and PBMCs from AAA patients (n=6). The frequency of IL-17A⁺ T cells among Vδ2⁺ T cells was examined. A representative FACS plot (left panel). Summary data (right panel). (D, E) Summary data of IFN-γ⁺ cells and TNF-α⁺ cells among each T-cell subset from aortic aneurysmal tissue (n=6) (left panel). Summary data of the IFN-γ⁺ cells and TNF-α⁺ cells among Vδ2⁺ T cells in aortic tissue and PBMCs from AAA patients (n=6) (right panel). (F, G) Summary data of the granzyme B⁺ cells and perforin⁺ cells among each T-cell subset from aortic aneurysmal tissue (n=6) (left panel). Summary data of the granzyme B⁺ cells and perforin⁺ cells among Vδ2⁺ T cells in aortic tissue and PBMCs from AAA patients (n=6) (right panel). Data are presented as mean and SD. Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test or Mann-Whitney *U* test.
N.S., not significant.
*p<0.05; **p<0.01.

In apolipoprotein E knock-out (ApoE-KO) mice, Vu et al. (25) demonstrated that γδ T-cell depletion suppresses early lipid accumulation in the intimal layer, in association with the infiltration of inflammatory cells. Another previous study suggests that γδ T cells may augment apoptosis of the cells constituting the aortic wall by suppressing the genes involved in phosphoinositide 3-kinase signaling and proliferation (*Sos1*, *Mtor*, and *Myc*) (26). In

accordance with these previous studies, our present analysis of human aortic aneurysm tissue demonstrated that Vδ2⁺ T cells were predominantly observed in aortic aneurysm tissue compared to in the peripheral blood of aneurysm patients or normal aortic tissue and the phenotype of most Vδ2⁺ T cells were effector memory cells. This abundance of effector memory Vδ2⁺ T cells reinforces the widely accepted concept that adaptive immunity plays a pivotal role in AAA pathogenesis and progression (8).

In our present study, immunological characterization revealed that Vδ2⁺ T cells prominently expressed IL-17A. IL-17A is a pro-inflammatory cytokine that is known to be mainly produced by Th17 cells (27). However, in the current study, we found that Vδ2⁺ T cells is the main source of IL-17A in aortic aneurysm tissue from AAA patients. IL-17A was a representative cytokine known to be involved in AAA pathogenesis through the regulation of inflammatory responses and extra-cellular matrix degradation (28). In addition, it has been suggested that IL-17A induced many other cytokines, including IL-1, IL-6, TNF- α, chemokines and inducible nitro oxide (27). Notably, expressions of cytotoxic enzymes and cytokines were suppressed in the Vδ2⁺ T cells of aortic aneurysm tissue. Also, we found the increased CXCR5 expression in Vδ2⁺ T cells in aneurysmal tissue. In a previous study showed that CXCR5⁺ Vδ2⁺ T cells have more effector functions than CXCR5⁻ Vδ2⁺ T cells (13). This study supported our suggestion that increased CXCR5 expression in Vδ2⁺ T cells might be associated with IL-17A production by Vδ2⁺ T cells in aneurysmal tissue. Taken together, these findings suggest that the distinct expression of Vδ2⁺ T cells in aortic aneurysm tissue, which retained robust memory function and IL-17A production, may orchestrate the inflammatory responses in the pathogenesis of aortic aneurysm. Further research is required to precisely identify the detailed molecular and cellular mechanisms through which IL-17A-producing Vδ2⁺ T cells in aortic aneurysm tissue are involved in AAA pathogenesis and progression.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1
Gating strategy for Vδ2⁺ T cells or Vδ1⁺ T cells and the frequency of regulatory T cells. (A) Gating strategy for Vδ2⁺, Vδ1⁺, CD4⁺ and CD8⁺ T cells was shown by representative FACS plots. (B) The frequency of regulatory T cells was examined and summary data presented. Data are presented as mean and SD. Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test or Mann-Whitney *U* test.

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Supplementary Figure 2
Vδ2⁺ T cells are the main source of IL-17A in aortic aneurysmal tissue with anti-CD3 stimulation. (A-E) IL-17A, IFN-γ, TNF-α, perforin and granzyme B were analyzed by flow

cytometry with anti-CD3 stimulation. (A) Flow cytometry analysis of IL-17A⁺ cells among CD4⁺, CD8⁺, and Vδ2⁺ T cells in aortic tissue from AAA patients (n=6) and Vδ2⁺ T cells in PBMCs with anti-CD3 stimulation. The frequency of IL-17A⁺ T cells in each T-cell subset was examined. (B, C) Summary data of IFN-γ⁺ cells and TNF-α⁺ cells among each T-cell subset from aortic aneurysmal tissue (n=6) (left panel). Summary data of the IFN-γ⁺ cells and TNF-α⁺ cells among Vδ2⁺ T cells in aortic tissue and PBMCs from AAA patients (n=6) (right panel). (D, E) Summary data of the granzyme B⁺ cells and perforin⁺ cells among each T-cell subset from aortic aneurysmal tissue (n=6) (left panel). Summary data of the granzyme B⁺ cells and perforin⁺ cells among Vδ2⁺ T cells in aortic tissue and PBMCs from AAA patients (n=6) (right panel). Data are presented as mean and SD. Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test or Mann-Whitney *U* test.

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