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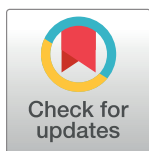
RESEARCH ARTICLE

Clonally expanded alpha-chain T-cell receptor (TCR) transcripts are present in aneurysmal lesions of patients with Abdominal Aortic Aneurysm (AAA)

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Abstract

Abdominal aortic aneurysm (AAA) is a life-threatening immunological disease responsible for 1 to 2% of all deaths in 65 year old or older individuals. Although mononuclear cell infiltrates have been demonstrated in AAA lesions and autoimmunity may be responsible for the initiation and account for the propagation of the disease, the information available about the pathogenesis of AAA is limited. To examine whether AAA lesions from patients with AAA contain clonally expanded α -chain TCR transcripts, we amplified by the non-palindromic adaptor-PCR (NPA-PCR)/ $V\alpha$ -specific PCR and/or the $V\alpha$ -specific PCR these α -chain TCR transcripts. The amplified transcripts were cloned and sequenced. Substantial proportions of identical α -chain TCR transcripts were identified in AAA lesions of 4 of 5 patients, demonstrating that clonally expanded T cells are present in these AAA lesions. These results were statistically significant by the bimodal distribution. Three of 5 of these patients were typed by DNA-based HLA-typing and all three expressed DRB1 alleles containing the DR β Gln70 amino acid residue that has been demonstrated to be associated with AAA. All three patients exhibited clonally expanded T cells in AAA lesions. Four of the 5 patients with AAA who exhibited clonal expansions of α -chain TCR transcripts, also exhibited clonal expansions of β -chain TCR transcripts in AAA lesions, as we have demonstrated previously (J Immunol 192:4897, 2014). $\alpha\beta$ TCR-expressing T cells infiltrating AAA lesions contain T-cell clones which have undergone proliferation and clonal expansion *in vivo* in response to as yet unidentified specific antigens that may be self or nonself. These results

provide additional evidence supporting the hypothesis that AAA is a specific antigen-driven T-cell autoimmune disease.

Introduction

Abdominal aortic aneurysm (AAA) is a common immunological disease with a strong genetic component diagnosed in 3% of 60 years old or older [1–6]. AAA accounts for 1–2% of deaths of 65 years old or older men, and is the 13th cause of death of men and women in the US of all ages [2]. AAA is characterized by dilations and enlargement of abdominal aorta with a diameter >3 cm or >50% of normal arteries [7]. Mortality of ruptured AAA is 85–90% [7,8].

Our understanding of the pathogenesis of AAA is limited. Environmental and genetic factors are involved and AAA is a complex multifactorial disease [3–5,9]. Strong evidence demonstrates that autoimmunity may be responsible for the pathogenesis of AAA and is summarized in Table 1 [3–5,9,10].

Our findings of substantial proportions of identical β -chain TCR transcripts in AAA lesions demonstrate the presence of clonally expanded T cells [10, 41]. These results are statistically significant, can be explained only by proliferation and clonal expansion *in vivo* of T cell clones in response to specific, as yet unidentified antigen(s) [10, 41], and strongly suggest that AAA is a specific antigen driven T cell disease [10,41].

Two highly polymorphic polypeptides, the α - and the β - chain, are used by the TCR to recognize peptides in association with self-MHC, and are rearranged exclusively in T cells [42]. We have shown that clonally expanded β -chain TCR transcripts are present in AAA lesions [10,41], however, this β -chain TCR clonality does not necessarily imply clonality of the α -chain TCR. Experimental proof is needed. The α -chain TCR clonality and repertoire should also be studied in AAA lesions for several reasons (Table 2).

Table 1. Evidence demonstrating that autoimmunity may be responsible for the pathogenesis of AAA.

	References
T, B, and NK cells and monocyte/macrophages infiltrate AAA lesions.	[10–12]
Mononuclear infiltrating cells express early (CD69), intermediate (CD38, CD25) and late (CD45RO, HLA Class II) activation antigens revealing an ongoing immune response.	[10]
APCs are present in AAA lesions, often in physical contact with CD4+, CD8+ and B-lymphocytes.	[3, 13, 14]
Pro-inflammatory Th1 cytokines (IFN- γ , IL-2, and others) play an important role in the destruction of the aortic wall and the pathogenesis of AAA. Th2 cytokines are also present in AAA lesions.	[3, 9, 15–17]
IgG autoantibody from AAA lesions recognize proteins present in normal aortic tissue.	[18, 19]
AAA is associated with HLA class I (HLA-A2, HLA-B61) and class II (HLA-DRB1*02, -DRB1*04).	[20–22]
Several putative AAA self-antigens have been identified including elastin and elastin fragments microbial-associated glycoprotein-36, collagen types I and III, carbonic anhydrase and oxidized low-density lipoprotein.	[3, 18, 19, 23–30]
Several putative AAA nonself (microbial) antigens have also been identified, including <i>Chlamydia pneumoniae</i> , <i>Treponema palladium</i> and cytomegalovirus. Molecular mimicry may be responsible for T-cell responses in AAA patients.	[31–36, 35, 37]
The frequency and the suppressor activity of CD4+CD25+FOXP3+ Tregs and the expression of FOXP3 transcript/protein are significantly lower in AAA vs normal donors.	[38–40]
Substantial proportions of identical β -chain T-cell receptor (TCR) transcripts are present in AAA lesions.	[10, 41]

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Table 2. Reasons to study α -chain TCR clonality and repertoire in AAA lesions.

	References
Both α - and β -chain TCR and in particular their CDR3s are critical in antigen recognition.	[43–46]
MHC class I & II restriction is mostly controlled by α -chain CDR1 and CDR3.	[42–49]
CD45RO ⁺ T cell clones specific for a single antigenic epitope employ a unique TCR β -chain and may be paired on the average with 25 different TCR α -chains; CD45RO ⁺ T-cell clones employ a TCR β -chain paired only with a single TCR α -chain	[50–52]
T cells expressing invariant α -chain TCR, but non-invariant β -chain TCR have been reported. Invariant natural killer T (NKT) cells utilize invariant α -chain TCR transcripts in both mice (V α 14J α 18) and humans (V α 24J α 18) and a limited number non-invariant β -chain TCR transcripts preferentially utilizing V β 2 or V β 7 (mice) and V β 11 (humans).	[53, 54]
HLA Class II DR α of DR molecule is monomorphic, while DQ α of DQ and DP α of DP molecules, respectively, are polymorphic. HLA class II are close counterparts of TCR in antigen recognition.	[55]
HLA class I are comprised of a polymorphic α -chain and an invariant β 2-microglobulin, which does not involve directly in antigen recognition but supports structurally the α -chain.	[55]
Expression in appropriate T cells of clonally expanded α - and β -chain TCR in AAA lesions and development of an antigen presentation system may permit the identification of putative AAA antigen (s).	[56]

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We report here that T cells infiltrating AAA lesions from patients with AAA contain clonally expanded α -chain TCR transcripts. Amplification of α -chain TCR transcripts from AAA lesions by the non-palindromic adaptor-PCR (NPA-PCR)/V α -specific PCR and/or the V α -specific PCR [41, 57–63] followed by cloning and sequencing demonstrated substantial proportions of identical α -chain TCR transcripts suggesting the presence of oligoclonal T cells. These results can be explained only by proliferation and clonal expansion of T-cell clones in vivo in response to specific, although not yet identified, self or non-self antigen(s), that they recognize.

Methods

Patients

The characteristics of the patients who provided AAA specimens for these studies are shown in Table 3. AAA size, race, age, gender, recent and past history of cardiovascular risk factors (high cholesterol, aortic valve replacement and current or ex-tobacco smoking) and associated diseases (chronic obstructive pulmonary disease, chronic renal disease, coronary artery disease, diabetes mellitus, and hypertension) are shown (Table 3). These patients were undergoing surgery for infrarenal AAAs repair. Adherent blood clots were stripped away from the aneurysm wall of these AAA specimens prior to use. Grossly normal infrarenal abdominal aortic specimens were obtained at autopsy from patients who died of non-vascular causes and employed as normal controls. Human peripheral blood mononuclear cells (PBMC) were employed as methodological control and were obtained from normal donors. The studies reported here were reviewed and approved by the IRB of Temple University Hospital and by the IRB of the Advocate Lutheran General Hospital. Written informed consent was obtained from the study participants. Procurement of additional peripheral blood from normal donors to prepare PBMC (methodological control) was approved by Old Dominion University.

Immunohistochemistry

AAA specimens were divided into two fractions. One was used for RNA preparation either immediately (fresh) or it was snap frozen in liquid nitrogen and used at a later time. The other

Table 3. Characteristics of the patients with AAA*.

Patient	Gender	Race	Age	AAA size (cm)	HTN	COPD	TOB	CHOL	DM	Other
AAA00	M	C	71	4.8	Y	N	N	N	N	
AAA03	M	C	80	5.5	Y	N	N	Y	N	
AAA09	M	C	78	7.4	Y	Y	N	N	N	CAD/ CRI
AAA10	M	C	78	7.9	N	N	N	Y	Y	CAD
AAA12	M	C	77	UN	UN	UN	UN	UN	UN	UN
	M = 5	C = 5	Avg. = xx n = 5	Avg. = xx n = 4 UN = 1	Yes = 3 No = 1 UN = 1	Yes = 1 No = 3 UN = 1	Yes = 0 No = 4 UN = 1	Yes = 2 No = 2 UN = 1	Yes = 1 No = 3 UN = 1	

M: Male; UN: Unknown; C: Caucasian; Y: Yes; N: No.

HTN: Hypertension; COPD: Chronic obstructive Pulmonary Disease; TOB: Current or ex- tobacco smoker; CHOL: High cholesterol; DM: Diabetes mellitus; CAD: Coronary artery disease; CRI: Chronic renal disease; AOVR: Aortic valve replacement.

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fraction was embedded in optimum cutting technology (OCT) formulation and it was snap frozen in liquid nitrogen and stored, until used, at -70°C for immunohistochemistry. Immunostaining was performed as described [64–66], by the avidin-biotin complex (ABC)—immunoperoxidase method (Vector Labs, Burlingame, CA), using: (i) an anti-CD3 monoclonal antibody (mab), clone NCL-CD3-PS1 (Novocastra, Newcastle upon Tyne, U.K.); (ii) an anti-CD4 mab clone 4B12 (Dako Glustrop, Denmark); (iii) an anti-CD8 mab clone C8/144B (Dako).

Isolation of PBMC from normal donors

PBMC were isolated from venous peripheral blood by a Ficoll-Hypaque density cushion [67].

DNA-based HLA-typing for HLA-DRB1, -DQA1 and -DQB1

DNA was prepared from AAA specimens for HLA-typing of DRB1, DQA1 and DQB1 loci as previously described [68]. Typing at HLA-DRB1 (exon 2) and DQB1 (exons 2 and 3) using AlleleSEQR typing reagents (Abbott Molecular, Des Plaines, IL). Typing at HLA-DQA1 was carried out using sequence specific primers (SSP) typing (Invitrogen SSP-Unitrays, Carlsbad, CA; Qiagen Olerup-SSP, Valencia, CA). Any remaining ambiguities of HLA-typing were resolved using sequence specific primers (SSP) typing (Invitrogen SSP-Unitrays, Carlsbad, CA; Qiagen Olerup-SSP, Valencia, CA). DNA sequencing was carried out using an ABI 3130 sequencer (Applied Biosystems, Carlsbad, CA). Results were analyzed by Assign-SBT v3.5 Software (Conexio Genomics, Fremantle, Australia).

RNA isolation

Total RNA was prepared from fresh (cryopreserved) AAA lesions containing tissue from these patients using a guanidinium thiocyanate solution, as recommended by the manufacturer (Stratagene, La Jolla, CA), and then treated with DNase from the Atlas pure total RNA labeling system (Clontech Laboratories, Inc., Mountain View, CA) to eliminate potential genomic DNA contamination.

Synthesis of cDNA

cDNA was synthesized from oligo-(dT)15- *NotI* (Promega) primed total RNA. We employed a SuperScript II (GibcoBRL) cDNA synthesis kit, according to the manufacturer's specifications [41,57–63]. Double-stranded cDNA was blunt-ended for efficient adaptor ligation by using T4 DNA polymerase.

Amplification by the nonpalindromic adaptor PCR (NPA-PCR)/ $V\alpha$ -specific PCR

(i) Adaptor ligation and *NotI* digestion. Double-stranded blunt-ended cDNA was ligated at the 5' and 3' blunt ends with an equivalent molar concentration of a nonpalindromic adaptor (NPA) comprised of two complementary oligonucleotides pre-annealed to each other: 5' -AATTCGAACCCCTTCGAGAATGCG-3' and 3' -GCTTGGGGAAGCTCTTACGC-p-5' (S1 Table). cDNA and NPA were ligated for 14 hrs at 16°C with T4 DNA ligase (Gibco-BRL). The ligated adaptor was removed from the 3' end of the double-stranded cDNA by *NotI* restriction endonuclease digestion (2 hrs at 37°C), while it was retained at the 5' end [41,57–63]. Digested cDNA was purified using a G-50 column as recommended (5'-3', Boulder, Co).

(ii) First cycle amplification by NPA-PCR. This was carried out as described [41,57–63], with minor modifications. The nonpalindromic adaptor 5' -AATTCGAACCCCTTCGAGA TGCT-3' oligonucleotide was employed as the 5' amplification primer. A hC α 3 oligonucleotide located in the C α region, 3' of J α , was employed as a 3' amplification primer (S1 Table). cDNA (20 μ l), purified by a G-50 column, was amplified using NPA-PCR, in 100 μ l, containing the cDNA, the amplification primers, 5 units of native pfu DNA polymerase and 1mM dNTPs in 1xbuffer [41,57–63]. PCR was performed, as follows: 5 min at 95°C for cDNA denaturation, amplification by 30 cycles including 1 min at 94°C (denaturation), 1 min at 45°C (annealing), 2 min at 72°C (elongation) and a final extension of 7 min at 72°C. The amplified transcripts were purified by a G-50 column, as recommended by the manufacturer.

(iii) Second cycle of amplification by individual $V\alpha$ -specific PCR. Thirty two different $V\alpha$ -specific PCRs were performed. NPA-PCR amplified α -chain TCR cDNA (4 μ l) was used as a template in a reaction volume of 50 μ l, which contained the cDNA, the amplification primers, 2.5 units of native pfu DNA polymerase and 1mM dNTPs in 1xbuffer [41,57–63]. Single oligonucleotides, each specific for one of 32 $V\alpha$ families (S1 Table), were used in 32 separate amplifications, as 5' end amplification primer. A C α 3' amplification primer designated as hC α 2 was used and it is located 5' to the hC α 3 primer employed for the first NPA-PCR amplification (nested design), 3' of J α (S1 Table). This nested design virtually eliminates possible PCR amplification of members of the Ig supergene family that may share homology with the α -chain TCR because it will be unlikely that these members will have substantial homology with both hC α 3 and hC α 2 sites. The reaction mixture was denatured at 95°C for 5 min, amplified by 30 PCR cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min followed by a 7 min final extension step at 72°C.

Single $V\alpha$ -specific PCR amplification

This was performed [41,57–63] to evaluate, in more detail, single $V\alpha$ families or subfamilies or to confirm clonal expansions identified by NPA-PCR/ $V\alpha$ -specific PCR. Template cDNA was synthesized from total RNA isolated from the same AAA specimen employed for NPA-PCR/ $V\alpha$ -specific PCR, or from total RNA prepared in separate experiments from the same AAA specimen. Selection of the $V\alpha$ families to be amplified by $V\alpha$ -specific PCR was made on the basis of the NPA-PCR/ $V\alpha$ -specific PCR findings. $V\alpha$ 5 and $V\alpha$ 24 (patient AAA03), $V\alpha$ 6, $V\alpha$ 9

and V α 12 (patient AAA09), V α 2 and V α 8 (patient AAA00) and V α 6 (patient AAA10) families were amplified by V α -specific PCR. V α family specific oligonucleotides (S1 Table), were used as 5' end amplification primers. The hC α 2 oligonucleotide was used as 3' amplification primer. V α -specific PCR (35 cycles), included denaturation (94°C, 1 min), annealing (55°C, 1 min), elongation (72°C, 2 min), and final extension (72°C, 7 min).

Cloning of the PCR products

Eight μ l from each one of the 32 NPA-PCR/V α -specific PCR amplified products were mixed together and incubated with Taq polymerase for 10 min at 72°C to add an adenine at the 3' end and the mixture was selected by agarose gel electrophoresis in respect to size and purified using a GeneClean Kit (Bio101, Vista, CA), following the instructions of the manufacturer. Mixing these PCR amplification products from each of the 32 V α families before cloning and sequencing, reduces substantially the work needed for these experiments [41,57–59]. Purified NPA-PCR/V α -specific PCR or V α -specific PCR products were cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA), were transformed into Top10 One Shot Chemically competent cells (Invitrogen) as described by the manufacturer and were submitted to blue-white screening. Top10 One Shot Chemically competent *E.coli* cells were incubated for 30–45 min with the vector on ice and were subjected to heat shock for 30–45 sec at 42°C. The competent cells were incubated on ice for 2 min and 250 μ l of SOC medium were added and incubated at 37°C for 1 hr. The competent cells were plated in X-gal containing agar plates [41,57–63]. White colonies were collected using the Perfectprep Plasmid Mini Kit (Eppendorf, Westbury, NY) following the specifications of the manufacturer. Large numbers of white colonies were obtained.

The TCR repertoire is very large [47–49,69,70] and for this reason the probability is very small to find by chance in an independent sample of T lymphocytes two identical copies of a single α - or β -chain TCR transcript. The only mechanism that can explain the presence of multiple identical copies of a single α - or β -chain TCR transcript in an independent population of T lymphocytes is specific antigen-driven proliferation and clonal expansion (reviewed in [56]). However, during transformation of DH5 α -*E. coli*-competent cells the mixture of *E. coli* cells and plasmid was heat shocked by treatment for 30 sec at 42°C followed by growth in SOC medium at 37°C for 1 hr before the colonies were plated. In ideal growth conditions (log phase growth) *E. coli* cells divide every 20 min and this could result in two divisions in one hour [71]. Because of the heat shock treatment, the *E. coli* cells may not enter right away to the logarithmic growth phase. However, it is possible, although unlikely, that few *E. coli*-transformed cells may double before they are plated. Therefore, the appearance in two different colonies of an identical TCR transcript, designated as a doublet, may indicate a clonal expansion or may be the result of a single *E. coli* cell transformed and divided (doubled) before it was plated. We are addressing this issue in the statistical method that we are employing (see below; second alternative hypothesis), although doubling of a transfected *E. coli* cell before plating is rather infrequent. As a methodological control, to make certain that all PCR, cloning and sequencing methods were performing correctly, we amplified by NPA-PCR/V α -specific PCR or NPA-PCR, cloned, and sequenced 125 α -chain TCR transcripts from PBMC of 3 normal donors. An additional 45 α -chain TCR transcripts from PBMC of 2 normal donors were reported previously (refs. [59] and [60]) (see below).

Sequencing

Plasmids were sequenced by the dideoxy chain termination method using 6% polyacrylamide DNA sequencing gels and an ABI373A DNA Sequencer (Applied Biosystems, Foster City,

CA). Comparable numbers of α -chain TCR clones were obtained after NPA-PCR/ $V\alpha$ -specific PCR amplification and cloning and as well as after $V\alpha$ -specific PCR and cloning.

Computer analysis. Sequence comparison

Sequences of α -chain TCR transcripts were identified in AAA lesions or in PBMC from normal controls encoding for V, D, J and C regions by comparing them to those in the NCBI databases by the standard nucleotide-nucleotide BLAST sequence alignment program [41,57–63]. The N region nucleotide sequence of TCR transcripts was identified as the nucleotide sequence contained between the last identifiable $V\alpha$ nucleotide and the first identifiable $J\alpha$ nucleotide. Deduced CDR3 region amino acid sequences were compared to those in the NCBI databases using BLAST gapped BLAST and PSI BLAST protein database programs.

Statistical analysis

The binomial distribution was employed [41,58,59,62] to determine the probability, p , that the number (x) of the multiple identical α -chain TCR transcripts identified among those sequenced (x/n ; n is the total number of α -chain TCR transcripts sequenced), was statistically significant versus (i) a first alternative hypothesis that each α -chain TCR transcript is expressed only once and all α -chain TCR transcripts sequenced when compared to each other are unique ($1/n$), or (ii) a second alternative hypothesis that only a single α -chain TCR transcript is expressed twice and all the remaining α -chain TCR transcripts identified are expressed only once ($2/n$) [41]. The first alternative and the second alternative hypotheses were initially developed [41] for the analysis of β -chain TCR, and their use is extended here to the analysis of α -chain TCR.

Results

Multiple identical copies of α -chain TCR transcripts are present in AAA lesions

Immunohistochemical staining of AAA tissues with the anti-CD3 mab revealed the presence of substantial CD3+ T-cell infiltrates in these AAA lesions which were predominant in the adventitia and media [10, 41]. Staining with the appropriate mabs revealed that these infiltrates contained both CD4+ and CD8+ T lymphocytes [41]. These results have been shown previously [10,41] and they are in agreement with the reports of others [11–14]. Rare, if any, mononuclear cell infiltrates are found in grossly normal autopsy infrarenal abdominal aortic tissue which was obtained from patients who died of nonvascular causes [41].

Multiple identical copies of α -chain TCR transcripts were found in AAA lesions from patients with AAA by PCR amplification, cloning and sequencing. Comparison of these sequences to those of the GenBank database using BLAST revealed that they were novel (not reported in the GenBank) and typical of productively rearranged human α -chain TCR transcripts. Statistically significant, by the binomial distribution, α -chain TCR clonal expansions were identified in AAA lesions in 4 of 5 patients with AAA.

Sequence analysis of α -chain TCR transcripts from AAA lesions from patient AAA03 after NPA-PCR/ $V\alpha$ -specific PCR amplification followed by cloning and sequencing revealed several clonal expansions (Table 4): (i) Clone aaa03npa04 accounted for 5 of 57 identical transcripts (9%) ($V\alpha 5.1J\alpha 3$) (CDR3: LE). These results are statistically significant by the bimodal distribution. Probability (p) of appearance of 5 of 57 identical α -chain TCR transcripts by chance against the alternative hypothesis that each α -chain TCR transcript is expressed only once $1/n = 1/57$, was $p = 0.003$. The p of the appearance of 5 of 57 identical α -chain TCR transcripts

Table 4. α -chain TCR transcripts (CDR3 region) expressed in aneurysmal wall of patients with AAA.

Clone	V α	N	J α	Transcript Frequency in Specimen	p value	
PATIENT AAA03						
α-chain TCR transcripts amplified by NPA- PCR/Vα-specific PCR					vs. 1/57	vs. 2/57
aaa03npa04	CA tgtgct	LE ctaga	GYSS ggggtacagcagt	V α 5.1J α 3 5/57(9%)	p = 0.003	p = 0.034
aaa03npa21	CVVS tgtgtggtgagc	AGR gcggttag	EYGNKL ggaatatggaaacaaactg	V α 24.1J α 47 5/57(9%)	p = 0.003	p = 0.034
aaa03npa09	CAY tgtgcttat	IS atctc	GNKL tggaacaaactg	V α 14.1J α 47 4/57(7%)	p = 0.015	p = 0.089
aaa03npa33	CAMR tgtgcaatgaga	EGE gagggcgag	GGSNYKL ggaggtagcaactataaactg	V α 6.1J α 54 4/57(7%)	p = 0.015	p = 0.089
aaa03npa16	CAV tgtgctgtg	E g	ETSGSRL aagaaaccagtggtcttaggtg	V α 13.1J α 58 3/57(5%)	p = 0.06	p = 0.183
aaa03npa02	CAG tgtgctggg	QK cagaag	GGTSYGKL ggtggtactagctatggaaagctg	V α 25.1J α 53 3/57(5%)	p = 0.06	p = 0.183
aaa03npa19	CAVE tgtgctgtggag	T acc	GFQKL ggctttcagaaactt	V α 11.1J α 8 2/57(4%)	ns	ns
aaa03npa12	CAV tgtgctgtc	VR gttc	NNNDRL ggaataacaatgacagactc	V α 13.1J α 31 2/57(4%)	ns	ns
aaa03npa10	CAV tgtgctgtg	VTSV gtcactagcgt	GSGNTGKL tggtctgtggcaacacaggcaacta	V α 13.1J α 37 2/57(4%)	ns	ns
aaa03npa26	CAV tgtgctgtg	AKD gcaaagg	GGSQGNL atggaggaagccaaggaaatctc	V α 13.1J α 42 2/57(4%)	ns	ns
aaa03npa11	CAV tgtgctgtg	EG gaggg	SSGDKL aagcagcggagacaagctg	V α 13.1J α 46 2/57(4%)	ns	ns
aaa03npa20	SAVY tctgcagtgtac	PSPVA ccatctcccgtcg	GGGNKL cgggaggaggaaacaaactc	V α 21.1J α 10 2/57(4%)	ns	ns
aaa03npa27	CAT tgtgctacg	DSFVYSN gactctttcgtttacagcaa	ASKI tgcttccaagata	V α 3.1J α 3 1/57(2%)	ns	ns
aaa03npa61	CAT tgtgctacg	AR gcc	MDSSYKL ggatggatagcagctataaattg	V α 3.1J α 12 1/57(2%)	ns	ns
aaa03npa36	CAT tgtgctacg	DA gacgca	NDYKL aacgactacaagctc	V α 3.1J α 20 1/57(2%)	ns	ns
Remaining 18 of 57 sequences are unique when compared to each other and are shown in the supporting information section, S2 Table .						
α-chain TCR transcripts amplified by single Vα5-specific PCR					vs. 1/20	vs. 2/20
aaa03npa04	CA tgtgct	LE ctaga	GYSSASKI ggggtacagcagtgcttccaagata	V α 5.1J α 3 20/20(100%)	p<0.0001	p<0.0001
α-chain TCR transcripts amplified by single Vα24-specific PCR					vs. 1/22	vs. 2/22
aaa03npa21	CVVS tgtgtggtgagc	AGR gcggttag	EYGNKL ggaatatggaaacaaactg	V α 24.1J α 47 13/22(59%)	p<0.0001	p<0.0001
aaa03va2401	CVVS tgtgtggtgagc		SGGSYI tcaggaggaagctacata	V α 24.1J α 6 6/22(27%)	p = 0.0003	p = 0.0092
aaa03va2408	CV tgtgtg	AA gcggc	TGGFKT tactggaggcttcaaaact	V α 24.1J α 9 3/22(14%)	p = 0.06	p = 0.189
PATIENT AAA09						

(Continued)

Table 4. (Continued)

Clone	V α	N	J α	Transcript Frequency in Specimen	p value	
					vs. 1/36	vs. 2/36
α-chain TCR transcripts amplified by NPA-PCR/Vα-specific PCR						
aaa09npa08	CALS	EPF	QTGANNL	V α 12.1J α 36	$p = 0.06$	$p = 0.187$
	tgtgctctgagt	gagccttt	tcaaaactggggcaaacaacctc	3/36(8%)		
aaa09npa16	CAL	SR	GSQGNL	V α 9.1J α 42	$p = 0.06$	$p = 0.187$
	tgtgctctg	tcta	gaggaagccaaggaaatctc	3/36(8%)		
aaa09npa01	CAMR	EGD	QAGTAL	V α 6.1J α 15	ns	ns
	tgtgcaatgaga	gagggag	accaggcaggaactgctctg	2/36(8%)		
aaa09npa37	CAMR	EIR	TSYDKV	V α 6.1J α 50	ns	ns
	tgtgcaatgaga	gagattcgg	acctcctacgacaaggtg	2/36(6%)		
aaa09npa22	CAL	RGL	NQAGTAL	V α 9.1J α 15	ns	ns
	tgtgctcta	aggggtctc	aaccaggcaggaactgctctg	2/36(6%)		
aaa09npa39	CAL	AP	SGNTGKL	V α 5.1J α 37	ns	ns
	tgtgctcta	gcccc	ctctggcaacacaggcaacta	1/36(3%)		
aaa09npa26	CAMR	S	NTGFQKL	V α 6.1J α 8	ns	ns
	tgtgcaatgaga	tc	gaacacaggctttcagaaactt	1/36(3%)		
aaa09npa09	CAMR	EGMA	GFQKL	V α 6.1J α 8	ns	ns
	tgtgcaatgaga	gagggcatgg	caggctttcagaaactt	1/36(3%)		
Remaining 21 of 36 sequences are unique when compared to each other and are shown in the supporting information section, S3 Table .						
α-chain TCR transcripts amplified by single Vα12-specific PCR					vs. 1/20	vs. 2/20
aaa09npa08	CALS	EPF	QTGANNL	V α 12.1J α 36	$p < 0.0001$	$p < 0.0001$
	tgtgctctgagt	gagccttt	tcaaaactggggcaaacaacctc	11/20(55%)		
aaa09va1201	CAL	RGT	NDYKL	V α 12.1J α 20	ns	ns
	tgtgctctg	aggggga	ctaacgactacaagctc	2/20(10%)		
aaa09va1207	CALS	AGAS	SSASKI	V α 12.1J α 3	ns	ns
	tgtgctctgagt	gcaggcgcgtc	cagcagtgcttccaagata	1/20(5%)		
aaa09va1219	CALS	RG	GNNRL	V α 12.1J α 7	ns	ns
	tgtgctctgagt	cgtgg	tgggaacaacagactc	1/20(5%)		
Remaining 5 of 20 sequences are unique when compared to each other and are shown in the supporting information section, S3 Table .						
α-chain TCR transcripts amplified by single Vα6-specific PCR					vs. 1/21	vs. 2/21
aaa09npa37	CAMR	EIR	TSYDKV	V α 6.1J α 50	$p = 0.06$	ns
	tgtgcaatgaga	gagattcgg	acctcctacgacaaggtg	3/21(14%)		
aaa09va0609	CAMR	EGL	YGGATNKL	V α 6.1J α 32	ns	ns
	tgtgcaatgaga	gagggcctc	tatggaggtgctacaacaagctc	2/21(10%)		
aaa09va0602	CAMR	G	GFGNVL	V α 6.1J α 35	ns	ns
	tgtgcaatgaga	ggg	ggctttgggaatgtgctg	2/21(10%)		
aaa09va0612	CAMR	EGP	GYSSASKI	V α 6.1J α 3	ns	ns
	tgtgcaatgaga	gagggccct	gggtacagcagtgcttccaagata	1/21(5%)		
aaa09va0608	CAM	SPNR	GGYNKL	V α 6.1J α 4	ns	ns
	tgtgcaatg	agccctaacagg	ggtggctacaataagctg	1/21(5%)		
aaa09va0607	CAMR	VNYRA	RRAL	V α 6.1J α 5	ns	ns
	tgtgcaatgaga	gtaaactatcggg	cgggcaggagagcactt	1/21(5%)		
Remaining 11 of 21 sequences are unique when compared to each other and are shown in the supporting information section, S3 Table .						
α-chain TCR transcripts amplified by single Vα9-specific PCR					vs. 1/19	vs. 2/19

(Continued)

Table 4. (Continued)

Clone	Vα	N	Jα	Transcript Frequency in Specimen	p value	
aaa09npa16	CAL	SR	GSQGNL	Vα9.1Jα42	<i>p</i> = 0.06	ns
	tgtgctctg	tcta	gaggaagccaaggaaatctc	3/19(16%)		
aaa09npa12	CAL	RFRA	YSSASKI	Vα9.1Jα3	ns	ns
	tgtgctctg	cgattccgtgcc	tacagcagtgcttccaagata	2/19(11%)		
aaa09va0906	CAL	S	GSGNTGKL	Vα9.1Jα37	ns	ns
	tgtgctcta	a	gtggctctggcaacacaggaacta	2/19(11%)		
aaa09npa24	CALS	RS GT	RL	Vα9.1Jα58	ns	ns
	tgtgctctaagt	cggtccggga	ctagggtg	2/19(11%)		
aaa09va0902	CALS	GD	NFNKF	Vα9.1Jα21	ns	ns
	tgtgctctaagt	gggg	acaacttcaacaaattt	1/19(5%)		
aaa09npa42	CA	QSP	GYNKL	Vα9.1Jα4	ns	ns
	tgtgct	cagtccc	ctgggtggctacaataagctg	1/19(5%)		
aaa09va0905	SA	SEGH	GSQGNL	Vα9.1Jα42	ns	ns
	tcagcc	tcagaaggtc	atggaggaagccaaggaaatctc	1/19(5%)		

Remaining 7 of 19 sequences are unique when compared to each other and are shown in the supporting information section, S3 Table.

PATIENT AAA00

α-chain TCR transcripts following Vα2-specific PCR				vs.	vs.	
				1/15	2/15	
aaa00va0201	CVV	TG	TGGFKT	Vα2.3Jα9	<i>p</i> < 0.0001	<i>p</i> < 0.0001
	tgtgtggtg	acggga	actggaggcttcaaaact	15/15 (100%)		

α-chain TCR transcripts following Vα8-specific PCR

				vs.	vs.	
				1/15	2/15	
aaa00va0801	CAE	E	GSNYKL	Vα8.2Jα54	<i>p</i> < 0.0001	<i>p</i> < 0.0001
	tgtgcagag	gag	ggaggtagcaactataaactg	15/15 (100%)		

PATIENT AAA10

α-chain TCR transcripts amplified by NPA-PCR/Vα-specific PCR				vs.	vs.	
				1/30	2/30	
aaa10npa03	CAM	TPPG	GGTSYGKL	Vα6.1Jα53	<i>p</i> = 0.06	ns
	tgtgcaatg	acacctcccggg	ggtggtactagctatggaaagctg	3/30(10%)		
aaa10npa04	CAM	RET	NTDKL	Vα6.1Jα34	ns	ns
	tgtgcaatg	agagaaacg	aacaccgacaagctc	2/30(7%)		
aaa10npa07	CA	PFGG	SNSGYAL	Vα13.1Jα41	ns	ns
	tgtgct	cccttcggcggg	tcaaattccgggtatgcactc	2/30(7%)		
aaa10npa022	CGA	DYP	SGTYKY	Vα26.1Jα40	ns	ns
	tgtggagca	gactatc	cctcaggaacctacaaatac	2/30(7%)		
aaa10npa09	CAT	DY	GSARQL	Vα3.1Jα22	ns	ns
	tgtgctacg	gactac	tctggtcttgcaaggcaactg	1/30(3%)		
aaa10npa44	CAT	PK	SGTYKY	Vα3.1Jα40	ns	ns
	tgtgctacc	cctaag	tcaggaacctacaaatac	1/30(3%)		
aaa10npa43	CAT	DARH	SNSGYAL	Vα3.1Jα41	ns	ns
	tgtgctacg	gacgccgcc	actcaaattccgggtatgcactc	1/30(3%)		

Remaining 18 of 30 sequences are unique when compared to each other and are shown in the supporting information section, S4 Table.

α-chain TCR transcripts following Vα6-specific PCR				vs.	vs.	
				1/21	2/21	
aaa10npa03	CAM	TPPG	GGTSYGKL	Vα6.1Jα53	<i>p</i> = 0.01	<i>p</i> = 0.09
	tgtgcaatg	acacctcccggg	ggtggtactagctatggaaagctg	4/21(19%)		
aaa10npa29	CAMR	EAYS	GNQFY	Vα6.1Jα49	<i>p</i> = 0.01	<i>p</i> = 0.09

(Continued)

Table 4. (Continued)

Clone	Vα	N	Jα	Transcript Frequency in Specimen	p value	
	tgtgcaatgaga	gaggcgtact	ccggtaaccagttctat	4/21(19%)		
aaa10va0601	C A M R	E V D	T G G F K T	Vα6.1Jα9	ns	ns
	tgtgcaatgaga	gaggctcg	atactggaggcttcaaaact	2/21(10%)		
aaa10va0610	C A M R	E T	N T D K L	Vα6.1Jα34	ns	ns
	tgtgcaatgaga	gaaacg	aacaccgacaagctc	2/21(10%)		
aaa10va0614	C A M R	P R	S G Y S T L	Vα6.1Jα11	ns	ns
	tgtgcaatgaga	ccgag	gaattcaggatacagcaccctc	2/21(10%)		
aaa10va0620	C A M	S P	M D S S Y K L	Vα6.1Jα12	ns	ns
	tgtgcaatg	agtcc	gatggatagcagctataaattg	1/21(5%)		
aaa10va0618	C A M R	E A L	M D S S Y K L	Vα6.1Jα12	ns	ns
	tgtgcaatgaga	gaggccctt	atggatagcagctataaattg	1/21(5%)		
aaa10va0602	C A M R	D	G Q K L	Vα6.1Jα15	ns	ns
	tgtgcaatgaga	ga	tggccagaagctg	1/21(5%)		

Remaining 4 of 21 sequences are unique when compared to each other and are shown in the supporting information section, S4 Table.

PATIENT AAA12

α-chain TCR transcripts amplified by NPA-PCR/Vα-specific PCR				vs. 1/31	vs. 2/31
aaa12npa26	C A V	S A K	N N N A R L	Vα13.1Jα31	ns
	tgtgctgtg	tctgcgaa	gaataacaatgccagactc	2/31(6%)	
aaa12npa02	C A	P E G	G G G A D G L	Vα13.1Jα45	ns
	tgtgcc	ccggagggg	ggaggagggtgctgacggactc	2/31(6%)	
aaa12npa16	C G A	H S N	S G G G A D G L	Vα26.1Jα45	ns
	tgtggagcc	cactcaa	attcaggaggagggtgctgacggactc	2/31(6%)	
aaa12npa07	C A L	R	N Q T G T A L	Vα5.1Jα15	ns
	tgtgctctc	cgt	aaccagacaggaactgctctg	1/31(3%)	
aaa12npa04	C A L	E G D	N A G N M L	Vα5.1Jα39	ns
	tgtgctcta	gaagggg	ataatgcaggcaacatgctc	1/31(3%)	
aaa12npa28	C A M	T S K T I	I F G Q	Vα6.1Jα37	ns
	tgtgcaatg	acctctaaaacaa	taatctttgggcaa	1/31(3%)	

Remaining 22 of 31 sequences are unique when compared to each other and are shown in the supporting information section, S5 Table.

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against the alternative hypothesis that only a single α-chain TCR is expressed twice, 2/n = 2/57, and all remaining α-chain TCR transcripts sequenced were expressed only once, was p = 0.034; (ii) Clone aaa03npa21 accounted for 5 of 57 identical transcripts (9%)(p = 0.003 vs. 1/57 and p = 0.034 vs. 2/57)(Vα24.1Jα47) (CDR3: AGR); (iii) clone aaa03npa09 accounted for 4 of 57 identical transcripts (7%)(p = 0.015 vs. 1/57 and p = 0.089 vs. 2/57)(Vα14.1Jα47) (CDR3:IS); (iv) clone aaa03npa33 accounted for 4 of 57 identical transcripts (7%)(p = 0.015 vs. 1/57 and p = 0.089 vs. 2/57) (Vα6.1Jα54) (CDR3: EGE). (v) Clones aaa03npa16 and aaa03npa02 accounted each for 3 of 57 identical transcripts (p = 0.06); six clones were expressed in duplicate and the remaining 21 clones were unique when compared to each other. Eighteen of 57 of those clones are shown in the supporting information section (S2 Table).

The Vα5.1Jα3 clonal expansion (clone aaa03npa04)(CDR3:LE), identified by NPA-PCR/Vα-specific PCR, was confirmed by Vα5-specific PCR followed by cloning and sequencing (Table 4). Twenty of 20 (100%) (p<0.0001) Vα5.1Jα3 transcripts were identical to the clonally-expanded aaa03npa04 clone which was initially identified by NPA-PCR/Vα-specific PCR

(Table 4). The V α 24.1J α 47 clonal expansion (clone aaa03npa21) (CDR3: AGR) was confirmed by V α 24-specific PCR followed by cloning and sequencing. Thirteen of 22 (59%) ($p < 0.0001$) V α 24.1 transcripts were identical to the clonally expanded aaa03npa21 clone, initially identified by NPA-PCR/V α -specific PCR (Table 4). Another V α 24 clonal expansion was identified by V α 24-specific PCR followed by cloning and sequencing (clone aaa03Va2401) and accounted for 6 of 22 (27%) ($p = 0.0003$) identical transcripts (V α 24.1J α 6). Clone aaa03Va2408 (V α 24.1J α 9) was expressed in triplicate (Table 4).

Sequence analysis of α -chain TCR transcripts from AAA tissue from patient AAA09 after NPA-PCR/V α -specific PCR followed by cloning and sequencing revealed (Table 4) the presence of: (i) clone (aaa09npa08) that accounted for 3 of 36 identical transcripts (8%) (V α 12.1J α 36)(CDR3: EPF) ($p = 0.06$); (ii) clone (aaa09npa16) that accounted for 3 of 36 identical transcripts (8%) (V α 9.1J α 42) (CDR3:SR) ($p = 0.06$). Three α -chain TCR clones were expressed in duplicate. Remaining 24 of 36 α -chain TCR clones were unique when compared to each other and are shown in Table 4 (3 clones) and in the supporting information section (S3 Table)(21 clones).

The V α 12.1J α 36 clonal expansion identified by NPA-PCR/V α -specific PCR, was confirmed by V α 12-specific PCR followed by cloning and sequencing (Table 4). Eleven of 20 (55%) ($p < 0.0001$) V α 12.1J α 36 transcripts were identical to the clonally-expanded aaa03npa08 clone identified by NPA-PCR/V α -specific PCR (Table 4). One V α 12.1 clone was present in duplicate. Remaining 7 T-cell clones were unique when compared to each other and are shown in Table 4 and in S3 Table. Clone (aaa09npa23) identified (single copy) by NPA-PCR/V α .12.1-specific PCR (S3 Table) was also found (single copy) after V α 12.1-specific PCR (S3 Table).

Clone aaa09npa37, V α 6.1J α 50 (CDR3: EIR) was expressed in triplicate and accounted for 3 of 21 identical transcripts (14%) ($p = 0.06$) as determined by V α 6-specific PCR amplification, cloning and sequencing (Table 4). The same clone V α 6.1J α 50 was found to be expressed in duplicate (2 of 36; 6%; $p = 0.19$) by NPA-PCR/V α -specific PCR (Table 4). Remaining 14 V α 6.1 α -chain clones identified by V α 6-specific PCR, were unique when compared to each other and are shown in Table 4 (three) and S3 Table (eleven). However, three clones aaa09npa15, aaa09npa35 and aaa09npa45 were identified by both NPA-PCR/V α -specific PCR and V α 6-specific PCR followed by cloning and sequencing and were present in single copies (Table 4 and S3 Table).

Several clones identified by NPA-PCR/V α -specific PCR followed by cloning and sequencing were also identified by V α 9.1-specific PCR (Table 4). Clone aaa09npa16, V α 9.1J α 42 (CDR3:SR) accounted for 3 of 36 identical α -chain transcripts (8%) ($p = 0.06$) and was identified by NPA-PCR/V α 9.1-specific PCR. The same clone V α 9.1J α 42, CDR3:SR, was identified by V α 9.1-specific PCR and accounted for 3 of 19 identical α -chain TCR transcripts (16%) ($p = 0.06$). Clones aaa09npa16, aaa09npa24, aaa09npa42, and aaa09npa34 were identified by both NPA-PCR/V α -specific PCR and V α 9.1-specific PCR amplification, 3 out of 4 were present in single copies, and one in duplicate (Table 4 and S3 Table). Seven of 19 clones were unique when compared to each other and are shown in S3 Table.

V α 2-specific PCR amplification of TCR transcripts from AAA lesions from patient AAA00 followed by cloning and sequencing demonstrated 15 of 15 identical V α 2-chain TCR transcripts (100%) ($p < 0.0001$)(clone aaa00va0201)(V α 2.3Ja9)(Table 4). V α 8-specific PCR of TCR transcripts from AAA lesions from patient AAA00, followed by cloning and sequencing, revealed 15 of 15 identical V α 8-chain TCR transcripts (100%)($p < 0.0001$)(clone aaa00va0801) (V α 8.2J α 54) (Table 4).

Sequence analysis of α -chain TCR transcripts from AAA lesions from patient AAA10 after NPA-PCR/V α -specific PCR amplification and cloning, showed 3 of 30 (10%) identical α -chain TCR transcripts (clone aaa10npa03)($p = 0.06$)(V α 6.1J α 53)(CDR3:TPPG)(Table 4).

Clones, aaa10npa04 (V α 6.1J α 34), aaa10npa07 (V α 13.1J α 41), and aaa10npa22 (V α 26.1J α 40) were expressed in duplicate. Remaining α -chain transcripts were unique when compared to each other and are shown in Table 4 (three clones) and S4 Table (15 clones).

The V α 6.1J α 53 clonal expansion, identified by NPA-PCR/V α -specific PCR amplification was confirmed by V α 6-specific PCR followed by cloning and sequencing (Table 4). Four of 21 identical TCR transcripts (19%) ($p = 0.01$) (clone aaa10npa03)(V α 6.1J α 53)(CDR3:TPPG) identified by V α 6-specific PCR, cloning and sequencing, were identical to clone aaa10npa03, V α 6.1J α 53, CDR3:TPPG, identified by NPA-PCR/V α -specific PCR (3 of 30 identical transcripts) (Table 4 and S4 Table).

V α 6-specific PCR amplification, cloning and sequencing showed an additional clonal expansion of 4 of 21 identical TCR transcripts (19%) ($p = 0.01$) (clone aaa10npa29)(V α 6.1J α 49)(CDR3:EAYS)(Table 4). This aaa10npa29 clone was also identified after NPA-PCR/V α -specific PCR (single transcript, 1 of 30). Three clones amplified by V α 6-specific PCR were expressed in duplicate and 7 clones were expressed in single copies (Table 4 and S4 Table).

Sequence analysis of α -chain TCR transcripts after NPA-PCR/V α -specific PCR amplification and cloning from AAA lesions from patient AAA12, revealed 3 clones aaa12npa26 (V α 13.1J α 31), aaa12npa02 (V α 13.1J α 45) and aaa12npa16 (V α 26.1J α 45) expressed in duplicate (2 of 31; 6%; $p = 0.19$)(Table 4). Remaining 25 α -chain TCR transcripts were unique when compared to each other and are shown in Table 4 (three clones) and S5 Table (nineteen clones). Further analysis of TCR transcripts from patient AAA12 was not carried out.

Grossly normal autopsy specimens of infrarenal abdominal aortas from 3 patients who died of nonvascular diseases were used as controls. RNA was prepared and β -chain TCR NPA-PCR/V β -specific PCR revealed the absence of β -chain TCR transcripts and of infiltrating T cells in these non-aneurysmal aortic tissue specimens [41]. These findings are in agreement with the reports of others that T cells or CD45+ cells are absent in nonaneurysmal aortic tissue [7,72,73]. Along these lines, we have reported the absence of infiltrating T cells from central epicardial arteries [61].

PBMC from normal donors (methodological controls) are comprised of polyclonal T cells

We employed PBMC from 3 normal donors as methodological controls to ensure that all methods used in this study were performing well, as expected. Sequence analysis of α -chain TCR transcripts after NPA-PCR/V α -specific PCR and cloning demonstrated that these transcripts were productively rearranged human α -chain TCR transcripts and typical of polyclonal T cells (Table 5, normal donor 1) (S6 Table), in agreement with our previous findings [59,60]. A total of 170 α -chain TCR transcripts were sequenced from PBMC from normal donors (Table 5, S6 Table, and [59,60]) and all were unique when compared to each other with the exception of 12 of 170 transcripts (7%), which appeared in duplicate: (i) 2 of 28, normal donor 1 (Table 5); (ii) 2 of 47, normal donor 2 (S6 Table); (iii) 6 of 50, normal donor 3 (S6 Table); (iv) 1 of 25 [59]; and (v) 1 of 20 [60]. As it was mentioned in Methods (above), appearance in two different colonies of an identical α -chain TCR transcript, designated as a doublet, may indicate the beginning of a clonal expansion or may be the result of an artifact of the *E. coli* transfection method, and in particular of a single *E. coli* cell that was transformed and divided (doubled) before plating.

Control studies using PBMC from normal donors demonstrate that these results reveal true clonal expansions of T cells

The clonal expansions reported here were obtained by amplifying TCR transcripts by two PCR methods. An argument could be made that in the event that these two PCR amplifications

Table 5. α -chain TCR Transcripts (CDR3 Region) Identified in PBMC from Normal Donors.

Clone	V α	N	J α	Transcript Frequency in Specimen	p value	
					vs. 1/28	vs. 2/28
α-chain TCR transcripts from Normal Donor 1 amplified by NPA-PCR/Vα-specific PCR						
NBAnpa09	C A M R tgtgcaatgaga	E G R V gagggccgtgt	G T A S K L cggcactgccagtaaactc	V α 6.1J α 44 2/28	ns	ns
NBAnpa12	C A L tgtgctctg	R G agggggt	L I K A A G N K L tgatcaaagctgcaggcaacaagcta	V α 12.1J α 17 1/28	ns	ns
NBAnpa28	C A V tgtgccgtg	N R aata	G Y Q K V ggggttaccagaaagtt	V α 2.1J α 13 1/28	ns	ns
NBAnpa02	C A T tgtgctacg	E G V gagggggt	D Y K L cgactacaagctc	V α 3.1J α 20 1/28	ns	ns
NBAnpa16	C A M R tgtgcaatgaga	A G P gccggtc	G T A L caggaactgctctg	V α 6.1J α 15 1/28	ns	ns
NBAnpa07	C A M R tgtgcaatgaga	E G G gagggcg	D N Y G Q N F gggataactatggtcagaatttt	V α 6.1J α 26 1/28	ns	ns
NBAnpa36	C A L tgtgctcta	K A aagg	G G S Y I P caggaggaagctacatacct	V α 9.1J α 6 1/28	ns	ns
NBAnpa05	C A G tgtgcagga	A V P K gctgtcccca	Y G N K L aataatggaaacaaactg	V α 10.1J α 47 1/28	ns	ns
NBAnpa03	C A L S tgtgctctgagt	F tt	N A G N R R K L taatgctggcaacaaccgtaagctg	V α 12.1J α 38 1/28	ns	ns
NBAnpa34	C A V tgtgctgtg	T T acaacg	T G A N S K L actggagccaatagtaagctg	V α 13.1J α 56 1/28	ns	ns
NBAnpa23	C A V tgtgctgtg	T R acacga	T G A N I K L actggagccaatattaagctg	V α 13.1J α 56 1/28	ns	ns
NBAnpa08	C A E S tgtgcagagagt	I S ataag	S S A S K I cagcagtgtctccaagata	V α 15.1J α 3 1/28	ns	ns
NBAnpa27	C A E S tgtgcagagagt	S L tccctt	N T G G F K T aatactggaggcttcaaaact	V α 15.1J α 9 1/28	ns	ns
NBAnpa22	C A E tgtgcagag	L N ctaa	Q A G T A L accaggcaggaactgctctg	V α 15.1J α 15 1/28	ns	ns
NBAnpa21	C A E S tgtgcagagagt	M T atgacg	A A G N K L gctgcaggcaacaagcta	V α 15.1J α 17 1/28	ns	ns
NBAnpa32	C A A S tgtgcagcaagc	R M agaa	D S N Y Q L tggatagcaactatcagtta	V α 17.1J α 33 1/28	ns	ns
NBAnpa37	C A V tgtgctgtc	R Y L G G agatatttaggggt	G A T N K L ggtgtacaaaacaagctc	V α 19.1J α 32 1/28	ns	ns
NBAnpa29	C A V tgtgctgtc	V M gtgatg	Y G N K L tatgaaacaagctg	V α 19.1J α 47 1/28	ns	ns
NBAnpa25	C L V tgcctcgtg	G P F ggtccctt	N N A R L taacaatgccagactc	V α 20.1J α 31 1/28	ns	ns
NBAnpa15	C A G tgtgctggg	Q L D cagctgg	N T D K L ataacaccgacaagctc	V α 25.1J α 34 1/28	ns	ns
NBAnpa20	C A G tgtgctggt	L ct	S G T Y K Y ctcaggaacctacaaatac	V α 25.1J α 40 1/28	ns	ns
NBAnpa35	C A G tgtgctggg	P R ccgagg	T G T A S K L accggcactgccagtaaaactc	V α 25.1J α 44 1/28	ns	ns
NBAnpa33	C G A D	R G	D S S Y K L	V α 26.1J α 12	ns	ns

(Continued)

Table 5. (Continued)

Clone	V α	N	J α	Transcript Frequency in Specimen	p value	
	tgtggagcagac	cgagg	ggatagcagctataaattg	1/28		
NBAnpa30	CG	PLVPH	SGGGADGL	V α 26.1J α 45	ns	ns
	tgtgga	cccctcgtacctc	attcaggaggaggtgctgacggactc	1/28		
NBAnpa10	CRNL	LLHM	DTGRRAL	V α 30.1J α 5	ns	ns
	tgtaggaaccta	ctcctccaca	tggacacgggcaggagagcactt	1/28		
NBAnpa11	CLLG	STFY	NNNDM	V α 31.1J α 43	ns	ns
	tgtcttctggga	tctaccttct	acaataacaatgacatg	1/28		

Results from normal donors 2 and 3 are shown in the supporting information section S6 Table.

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were performed only from very few T cells, then it could be possible that each pair of amplification primers would amplify TCR transcripts from only few T cells, yielding findings that could resemble those shown in this paper. We have carried out extensive control experiments [41,58,59,62,63] using β -chain TCR transcripts demonstrating that this is not the case, that these results are true clonal expansions of T cells and they are not due to PCR amplification of TCR transcripts from just a few numbers of T cells. These results have been presented elsewhere [41,58,59,62,63] and will be briefly discussed here to address this point.

As it was mentioned above, each specimen containing AAA lesions from patients with AAA was divided into two halves. One half was employed for immunohistochemistry and the other half for RNA preparation, and TCR amplification, cloning and sequencing. The yield of RNA was approximately 10 μ g per preparation, which represents approximately 1.0×10^7 cells. An amount of 50 ng of RNA was used for each PCR amplification, cloning and sequencing. It is estimated that these 50 ng of RNA are isolated from approximately 5.0×10^4 cells.

The representation (ratio) of different TCR clones is the same in a sample of 10 μ g RNA and a sample of 50 ng RNA that we employed for PCR, cloning, and sequencing. The clonally expanded TCR transcripts that were identified in 10 μ g RNA, are also present in 50 ng RNA; the ratio of the different clonally expanded TCR transcripts to each other does not change. However, when different amounts of RNA are used in the PCR amplification the absolute number of copies of the clonally expanded TCR transcripts that are present is different.

We employed an anti-CD3 mab and immunohistochemical staining to determine the numbers of CD3+ T lymphocytes present in AAA specimens that we used for RNA preparation from patients AAA09 and AAA10. CD3+ T cells were counted in a large number (twenty) of high power fields per specimen by two different observers, independently. CD3+ T cell numbers varied substantially (range 0 to 155 CD3+ T lymphocytes) among each high power field. An average number of CD3+ T lymphocytes of approximately 780 per section and 660 per section were found in specimens AAA09 and AAA10, respectively. Because the thickness of aorta tissue specimens was approximately 5 mm and the thickness of the cryostat sections of the aorta specimens used were 6 μ m thick, the total number of CD3+ T lymphocytes employed for RNA isolation from aortic specimens from patients AAA09 and AAA10 were estimated to be 6.5×10^5 and 5.5×10^5 , respectively. In consideration that 10 μ g of RNA, derived from approximately 1×10^7 total cells present in these specimens, was recovered per preparation, CD3+ T lymphocytes alone in the AAA09 and AAA10 specimens were 6.5% and 5.5%, respectively (mean 6%), of the total cells employed to isolate RNA. An amount of 50 ng of RNA, representing approximately 5×10^4 cells, were employed for PCR amplification. CD3+ T lymphocytes accounted for about 6% of these cells, i.e., approximately 3,000 T cells.

Further control experiments were performed to identify the threshold of the minimum number of CD3⁺ T lymphocytes present in normal donor PBMCs that will provide polyclonal TCR transcripts after two PCR cycles, cloning and sequencing. Sequence analysis, after NPA-PCR amplification with various amounts of cDNA template and cloning, starting with as low as 300 T cells [41], demonstrated the presence of unique transcripts when compared with each other, except of two TCR transcripts which were present in duplicate (statistically not significant), typical of polyclonal T cells. The numbers of these T lymphocytes were 10 times lower than those present in AAA specimens used in these experiments (i.e., 3,000 T lymphocytes, as determined by immunohistochemistry using an anti-CD3 mab, see above).

The same approach was used in connection with V β -specific PCR, followed by cloning and sequencing [41,58,59,62,63]. V β 2-specific PCR followed by cloning and sequencing of normal donor PBMC containing as low as 1,200 T cells and an estimated 100 V β 2⁺ T lymphocytes (in 50 ng RNA), demonstrated unique V β 2⁺ TCR transcripts when compared to each other with the exception of two TCR transcripts present in duplicate (not statistically significant), typical of polyclonal T lymphocyte populations. The T cell numbers, 1,200 T cells, used in these experiments were lower than those, 3,000 T cells, in 50 ng RNA from AAA specimens.

Sequence analysis after V β 2-specific PCR amplification and cloning, from another mixture that contained only 300 T cells from the peripheral blood of normal donors, corresponding to 24 V β 2⁺ T lymphocytes, showed a more restricted pattern, consisting of the following [41]: (i) one V β 2⁺ transcript in triplet; (ii) 4 transcripts in duplicate copies; and (iii) 8 other transcripts in a single copy. These clonal expansions are not statistically significant. These findings confirm that the clonal expansions of T lymphocytes identified in AAA lesions represent real clonal expansions and are not due to amplifications of TCR transcripts from just a few T cells.

DNA-based HLA-typing for HLA-DRB1, -DQA1 and -DQB1

Three of 5 patients with AAA were typed by DNA-based HLA-typing for HLA-DRB1, -DQA1 and -DQB1 (Table 6). All three patients, AAA03, AAA09 and AAA10, expressed DRB1 alleles positive for the DR β Gln70 amino acid residue, which was reported to be associated with AAA [21]. Clonally expanded T cells in AAA lesions were present in all these 3 patients (Table 4). DNA-based HLA-typing of six patients with AAA, including the three patients shown here, has been reported previously [41], and is shown here to present a complete picture.

Conserved CDR3 amino acid motifs

A substantial number of CDR3 amino acid motifs were found in higher proportions in the TCR CDR3 from AAA lesions of patients with AAA vs. those of PBMC from normal donors, which were used as methodological controls (Table 7). These CDR3 amino acid motifs were selected with two amino acids each, because examination of the CDR3 sequences identified in

Table 6. DNA-based typing for HLA-DRB1, -DQA1 and -DQB1 loci of patients with AAA*.

Sample Name	DRB1-1	DRB1-2	DQA1-1	DQA1-2	DQB1-2	DQB1-2	DR β Q70
AAA03	0301	0101	0101	0501	0201	0501	+
AAA09	0101	0701	0101	0201	0202	0501	+
AAA10	0301	1501	0102	0501	0201	0602	+

* DNA-based HLA-typing results of these three patients with AAA have been reported previously [41] as part of a larger series and are shown here for the sake of completion. Reproduced in part, with the permission of The American Association of Immunologists, Inc., from Lu et al, J. Immunol., 192: 4897–4912, 2014 (reference [41]). Copyright 2014. The American Association of Immunologists, Inc.

Table 7. CDR3 α -chain TCR conserved amino acid motifs found in AAA lesions of patients with AAA.

CDR3 Motif	AAA Patient					Normal PBMC
	AAA03	AAA09	AAA00	AAA10	AAA12	
AG	26/99 (26%)	8/96 (8%)	0/30 (0%)	1/51 (2%)	2/31 (6%)	40/170 (23%)
AL	28/99 (28%)	48/96 (50%)	0/30 (0%)	2/51 (4%)	3/31 (10%)	25/170 (15%)
AY	6/99 (6%)	2/96 (2%)	0/30 (0%)	7/51 (14%)	0/31 (0%)	4/170 (2%)
EA	1/99 (1%)	2/96 (2%)	0/30 (0%)	6/51 (12%)	0/31 (0%)	2/170 (1%)
EE	4/99 (4%)	0/96 (0%)	15/30 (50%)	0/51 (0%)	6/31 (19%)	1/170 (0.5%)
EG	36/99 (36%)	11/96 (11%)	15/30 (50%)	2/51 (4%)	5/31 (16%)	9/170 (5%)
EP	0/99 (0%)	14/96 (15%)	0/30 (0%)	0/51 (0%)	0/31 (0%)	1/170 (0.5%)
GG	21/99 (21%)	23/96 (24%)	30/30 (100%)	30/51 (59%)	22/31 (71%)	42/170 (25%)
GGG	4/99 (4%)	2/96 (2%)	0/30 (0%)	8/51 (16%)	0/31 (0%)	8/170 (5%)
GR	18/99 (18%)	2/96 (2%)	0/30 (0%)	1/51 (2%)	0/31 (0%)	8/170 (5%)
GS	20/99 (20%)	17/96 (18%)	15/30 (50%)	5/51 (10%)	3/31 (10%)	35/170 (20%)
GT	5/99 (5%)	6/96 (6%)	15/30 (50%)	14/51 (27%)	2/31 (6%)	16/170 (9%)
LE	25/99 (25%)	0/96 (0%)	0/30 (0%)	0/51 (0%)	0/31 (0%)	9/170 (5%)
PF	0/99 (0%)	14/96 (15%)	0/30 (0%)	3/51 (6%)	0/31 (0%)	52/170 (30%)
PP	0/99 (0%)	0/96 (0%)	0/30 (0%)	7/51 (14%)	2/31 (6%)	0/170 (0%)
RE	23/99 (23%)	16/96 (17%)	0/30 (0%)	14/51 (27%)	1/31 (3%)	3/170 (2%)
RG	0/99 (0%)	18/96 (19%)	0/30 (0%)	0/51 (0%)	3/31 (10%)	2/170 (1%)
SA	39/99 (39%)	10/96 (10%)	0/30 (0%)	0/51 (0%)	3/31 (10%)	6/170 (3%)
SE	0/99 (0%)	20/96 (21%)	0/30 (0%)	0/51 (0%)	0/31 (0%)	14/170 (8%)
SG	17/99 (17%)	19/96 (20%)	0/30 (0%)	18/51 (35%)	4/31 (13%)	32/170 (19%)
SS	35/99 (35%)	8/96 (8%)	0/30 (0%)	3/51 (6%)	5/31 (16%)	7/170 (4%)
SY	10/99 (10%)	4/96 (4%)	0/30 (0%)	10/51 (20%)	3/31 (10%)	29/170 (17%)
TG	7/99 (7%)	24/96 (25%)	30/30 (100%)*	6/51 (12%)	0/31 (0%)	9/170 (5%)
TP	1/99 (1%)	1/96 (1%)	0/30 (0%)	8/51 (16%)	0/31 (0%)	20/170 (12%)
VS	24/99 (24%)	1/96 (1%)	0/30 (0%)	0/51 (0%)	5/31 (16%)	27/170 (16%)
VT	2/99 (2%)	1/96 (1%)	15/30 (50%)	1/51 (2%)	1/31 (3%)	2/170 (1%)
VV	28/99 (28%)	1/96 (1%)	15/30 (50%)	0/51 (0%)	5/31 (16%)	13/170 (8%)
YS	26/99 (26%)	6/96 (6%)	0/30 (0%)	8/51 (16%)	0/31 (0%)	5/170 (3%)

*Each one of 15 transcripts expressed 2 TG amino acid motifs (30 transcripts were sequenced from patient AAA00).

Statistical Analysis: **AG**: AAA vs. Normal PBMC, **p = 0.0034**; **AL**: AAA vs. Normal PBMC, **p = 0.0106**; **AY**: AAA vs. Normal PBMC, **p = 0.1941**; **EA**: AAA vs. Normal PBMC, **p = 0.2398**; **EE**: AAA vs. Normal PBMC, **p = 0.0100238**; **EG**: AAA vs. Normal PBMC, **p < 0.0001**; **EP**: AAA vs. Normal PBMC, **p = 0.0479**; **GG**: AAA vs. Normal PBMC, **p = 0.0044**; **GGG**: AAA vs. Normal PBMC, **p = 0.9435**; **GR**: AAA vs. Normal PBMC, **p = 0.3680**; **GS**: AAA vs. Normal PBMC, **p = 0.8067**; **GT**: AAA vs. Normal PBMC, **p = 0.2030**; **LE**: AAA vs. Normal PBMC, **p = 0.2680**; **PF**: AAA vs. Normal PBMC, **p < 0.0001**; **PP**: AAA vs. Normal PBMC, **p = 0.1276**; **RE**: AAA vs. Normal PBMC, **p = 0.0001**; **RG**: AAA vs. Normal PBMC, **p = 0.0174**; **SA**: AAA vs. Normal PBMC, **p = 0.0002**; **SE**: AAA vs. Normal PBMC, **p = 0.5012**; **SG**: AAA vs. Normal PBMC, **p = 0.9867**; **SS**: AAA vs. Normal PBMC, **p = 0.0005**; **SY**: AAA vs. Normal PBMC, **p = 0.0132**; **TG**: AAA vs. Normal PBMC, **p < 0.0001**; **TP**: AAA vs. Normal PBMC, **p = 0.0009**; **VS**: AAA vs. Normal PBMC, **p = 0.0671**; **VT**: AAA vs. Normal PBMC, **p = 0.0210**; **VV**: AAA vs. Normal PBMC, **p = 0.0183**; **YS**: AAA vs. Normal PBMC, **p = 0.0017**.

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AAA lesions revealed the presence of CDR3 motifs comprised of two amino acids each that were expressed in high proportions in more than one patients with AAA. The proportions of these CDR3 two amino acid motifs in patients with AAA were compared to the proportions of these CDR3 two amino acid motifs in PBMC from normal donors (Table 7) and were statistically significant by the differences in Poisson rates. The percent proportions of 17 of 28 CDR3

two amino acid motifs identified in AAA lesions were statistically significant in comparison to the percent proportions of these CDR3 two amino acid motifs expressed in PBMC from normal donors. We have previously used this approach to analyze CDR3 motifs [41]. Certain α -chain TCR CDR3 two amino acid motifs were found in increased proportions in more than one AAA patient (Table 7). Different α -chain TCR CDR3 amino acid motifs were utilized by different patients with AAA, suggesting the recognition of different peptide/MHC complexes by clonally expanded T cells or other T-cell clones present in AAA lesions from different patients. A total of 170 α -chain TCR transcripts from PBMC of 5 normal donors (Table 5 and [59,60]), were used as normal controls (Table 7).

Comparison of the nucleic acid and deduced amino acid sequences of α -chain TCR transcripts to those in the GENBANK/EMBL database

Comparison of all α -chain TCR sequences identified here to those in the GenBank/EMBL database by the BLAST software showed that the α -chain TCR transcripts identified in this study were novel and typical of α -chain TCR. None of the α -chain TCR transcripts reported here were reported previously. Identical α -chain TCR transcripts were not found in AAA lesions from different AAA patients. However, comparison of the CDR3 motifs of the clonally expanded α -chain TCR transcripts in AAA lesions by the gapped BLAST and PSI BLAST protein database programs revealed certain highly homologous CDR3s between α -chain TCR transcripts that were clonally expanded in AAA lesions to those previously reported in the GenBank/EMBL. Currently, it has not been established what is the maximum number of CDR3 amino acid differences that defines high or extensive CDR3 homology. We have chosen arbitrarily that differences of two conservative and one non-conservative amino acids would be the maximum number of differences allowed between CDR3 regions from different T-cell clones in order to define high or extensive CDR3 homology. Most important homologies include:

Patient AAA03 (Table 4): Clonally expanded clone aaa03npa04, CDR3: CALEGYSSASKI, exhibited extensive CDR3 homology with 3 T-cell clones: (i) CDR3: CALASYSSASKI, GenBank Accession No. ANO54287.1; (ii) CDR3: CALSYSSASKI, GenBank Accession No. ANO55176.1; (iii) CDR3: CAEKRGYSSASKI, GenBank Accession No. ANO54594.1; all identified in peripheral blood T cells of patients with Sjögren's syndrome (SS) [74]. Clone aaa03npa04 showed extensive CDR3 homology with a T-cell clone, CDR3: CAAPGYSNASKI, GenBank Accession No. AAA80058.1, found on anti-DNA antibody-helper T cells from the peripheral blood of a lupus patient [75]. Clone aaa03npa33, CDR3: CAMREGEGGSNYKL, showed high CDR3 homology with a T-cell clone, CDR3: CAVKEAGG GSNYKL, GenBank Accession No. ANO55390.1 found in peripheral blood T cells of an SS patient [74].

Clonally expanded clone aaa03npa02, CDR3: CAGQKGGTSYGKL, exhibited extensive CDR3 homology with 5 T-cell clones: (i) CDR3: CAAQGGTSYGKL, GenBank Accession No. ANO54008.1; (ii) CDR3: CAANAGGTSYGKL, GenBank Accession No. ANO55694.1; both identified in SG of patients with SS [74]; (iii) CDR3: CAGRNAGGTSYGKL, GenBank Accession No. ANO54097.1; (iv) CDR3: CAENGGTSYGKL, GenBank Accession No. ANO54594.1; (v) CDR3: CAGAPAGGTSYGKL, GenBank Accession No. ANO54605.1; all 3 identified in peripheral blood of patients with SS [74].

Clone aaa03npa16, CDR3: CAVEETSGSRL, had extensive CDR3 homology with 5 T-cell clones: (i) CDR3: CAVVEETSGSRL, GenBank Accession No. ANO54242.1; (ii) CDR3: CAVRETSGSRL, GenBank Accession No. ANO56281.1; (iii) CDR3: CAVDRETSGSRL, GenBank Accession No. ANO56267.1; all 3 found in salivary glands (SG) of SS patients [74]; (iv) CDR3: CAVKETSGSRL, GenBank Accession No. BAF94397.1, human T-cell clone [76]; (v) CDR3:

CAVRE TSGSRL, GenBank Accession No. AAB 97020.1; a human T-cell clone [77]. Clone aaa03va2408, CDR3: CVAATGGFKT, had extensive CDR3 homology with 3 T-cell clones: (i) CDR3: CAASTGGFKT, GenBank Accession No. ANO55378.1; (ii) CDR3: VADTGGFKT, GenBank Accession No. ANO 54837.1; both identified in SGs of SS patients [74]; (iii) CDR3: CAASTGGFKT, GenBank Accession No. AAC72697.1, human T-cell clone from the synovial fluid of a patient with rheumatoid arthritis [78].

Patient AAA09 (Table 4): Clonally expanded clone aaa10npa08, CDR3: CALSEPFQGTGAN NL, had extensive CDR3 homology with a T-cell clone, CDR3: EYAQTGANNL, GenBank Accession No. ANO56562.1, found in SG of an SS patient [74]. Clone aaa09npa16, CDR3: CALSRGSQGNL, showed extensive CDR3 homology with 5 T-cell clones: (i) CDR3: CALVRGSQGNL, GenBank Accession No. ANO56100.1; (ii) CDR3: CAMRDRSGSQGNL, GenBank Accession No. ANO55703.1; (iii) CDR3: CAVRRGSQGNL, GenBank Accession No. ANO55251.1; all 3 found in SGs of SS patients [74]; (iv) CDR3: CALGRNYGGSQGNL, GenBank Accession No. ANO54721.1; identified in peripheral blood T cells of an SS patient [74]; (v) CDR3: CAVIGRGSQGNL, GenBank Accession No. AAA80100.1; found on an anti-DNA antibody helper T-cell clone from peripheral blood of a lupus patient [75]; (vi) CDR3: CALSVGSQGNL, GenBank Accession No. AIE10490.1; found in a human T-cell clone [79]. Clone aaa 09npa37, CDR3: CAMREIRTSYDKV, had extensive CDR3 homology with a T-cell clone, CDR3: CAM REYPSYDKV, GenBank Accession No. ABO16436.1; found in a patient with renal cell carcinoma [80].

Patient AAA00 (Table 4): Clonally expanded clone aaa00va0201, CDR3: CVVTGTGGF KT, had extensive CDR3 homology with 3 T-cell clones: (i) CDR3: CVVNGAGGFKT, GenBank Accession No. ANO55862.1; (ii) CDR3: CVVSDGTGGFKT, GenBank Accession No. ANO56340.1; both identified in SGs of SS patients [74]; (iii) CDR3: CVVSEGTGGFKT, GenBank Accession No. ANO54058.1; found in peripheral blood T cells of an SS patient [74]. Clonally expanded clone aaa00va0801, CDR3: CAEEGGSNYKL, exhibited extensive CDR3 homology with: (i) CDR3: CAENRSGGSNYKL, GenBank Accession No. ANO55004.1; (ii) CDR3: CAENSSGGSNYKL GenBank Accession No. ANO55130.1; both identified in peripheral blood of an SS patient [74].

Patient AAA10 (Table 4): Clonally expanded clone aaa10npa03, CDR3:CAMTPPGGGT-SYGK had extensive CDR3 homology with a T-cell clone, CDR3: CAVSGPPAGGTSYGKL, GenBank Accession No. ANO54194; found in peripheral blood T cells of an SS patient [74]. Clone aaa10npa29, CDR3: CAMREAYSGNQFY, had extensive CDR3 homology with a T-cell clone, CDR3: CALSEANT GNQFY, GenBank Accession No. ANO55358; found in peripheral blood T cells of an SS patient [74].

A substantial number of clonally expanded α -chain TCR clones found in AAA lesions from patients with AAA, exhibited extensive CDR3 homology to T-cell clones found in SS patients.

Clonally expanded α - and β -chain TCR transcripts in AAA lesions of patients with AAA. Four of 5 patients who demonstrated in this study statistical significant clonal

Table 8. TCR Clonal Expansions and Oligoclonality of T Cells Infiltrating AAA Lesions.

Patients with AAA	Clonal Expansion/Oligoclonality	
	α -chain	β -chain
AAA03	Yes	Yes
AAA09	Yes	Yes
AAA00	Yes	Yes
AAA10	Yes	Yes
AAA12	Polyclonal	Polyclonal

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Table 9. Major Clonal Expansions of α - and β -chain TCR transcripts in AAA lesions.

Patient	α -chain % identical transcripts				β -chain % identical transcripts [41]			
	Transcript	Count	Percentage	p-value	Transcript	Count	Percentage	p-value
AAA09	V α 12.J α 36	11/20	55%	p<0.0001*	V β 14.1D β 2.1J β 2.3	12/21	57%	p<0.0001**
AAA00	V α 2.3J α 9	15/15	100%	p<0.0001*	V β 5.1D β 2.1J β 2.3	6/34	17.6%	p = 0.0004 ⁺
	V α 8.2J α 54	15/15	100%	p<0.0001*				
AAA03	V α 5.1J α 3	20/20	100%	p<0.0001*	V β 24.1D β 2.1J β 1.3 V β 6.3D β 2.1J β 2.1 V β 3.1D β 2.1J β 2.1	17/20	85%	p<0.0001** p = 0.01** p = 0.002**
	V α 24.1J α 47	13/22	59%	p<0.0001*		4/21 5/24	19% 21%	
AAA10	V α 6.1J α 53	4/21	19%	p = 0.01*	V β 3.1D β 2.1J β 2.1 V β 3.1D β 1.1J β 1.5	10/41 6/41	24% 15%	p<0.0001** p = 0.0004**
	V α 6.1J α 49	4/21	19%	p = 0.01*				
AAA12	Not significant				Not significant ⁺⁺			

* Clonally expanded α -chain TCR transcripts following V α -specific PCR amplification, cloning and sequencing (this report).

** Clonally expanded β -chain TCR transcripts following V β -specific PCR amplification, cloning and sequencing [41].

+ Clonally expanded β -chain TCR transcripts following NPA-PCR/V β -specific PCR amplification, cloning and sequencing [41].

++ Following NPA-PCR/V β -specific PCR amplification, cloning and sequencing [41].

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expansions of α -chain TCR transcripts in AAA lesions, also exhibited statistically significant clonal expansions of β -chain TCR transcripts. These results have been published previously [41] and are summarized here (Tables 8 and 9) for comparison purposes. In contrast, patient AAA12 exhibited polyclonal α - (Tables 4, 8 and 9) and β -chain [41] TCR transcripts in AAA lesions.

Discussion

To determine whether clonally-expanded α -chain TCR transcripts are present in T cells infiltrating AAA lesions from patients with AAA, we amplified α -chain TCR transcripts from these lesions by NPA-PCR/V α -specific PCR and cloned and sequenced the amplified transcripts. Analysis of the sequences demonstrated the presence of high proportions of identical α -chain TCR transcripts in 4 of 5 patients with AAA. These results were confirmed by two-sided V α -specific PCR, a independent amplification method, cloning and sequencing. Identical α -chain TCR clonal expansions to those obtained after NPA-PCR/V α -specific PCR were found. These results provide an important confirmation of the presence of oligoclonal T cells in AAA lesions and strongly support the view that AAA is a specific antigen-driven T-cell disease.

Previously, we reported that that β -chain TCR transcripts are clonally expanded in AAA lesions from 8 of 10 patients with AAA [41]. In this study, we demonstrate that α -chain TCR transcripts in AAA lesions are also clonally expanded. Four of 5 patients with AAA who exhibited statistically significant α -chain TCR clonal expansions in AAA lesions, also exhibited statistically significant β -chain TCR clonal expansions [41] (Tables 8 & 9). AAA lesions from patient AAA12 contained polyclonal α - and β -chain TCR transcripts (Tables 8 & 9). In the studies reported here we have continued the testing of the hypothesis that T cells infiltrating AAA lesions are oligoclonal and AAA is a specific antigen(s)-driven T-cell disease. To complete the testing of this hypothesis additional studies will be needed to identify the antigen(s), self or non-self, recognized by the clonally expanded α - and β -chain TCR.

Identical α -chain TCR transcripts were not identified in AAA lesions from different patients with AAA. This is in agreement with our results [41,57–63,80,81] and those of others [74–78,82] in a large number of studies demonstrating the absence of sharing among different patients with the same disease of clonally expanded identical α - and β -chain TCR transcripts. Similarly, entire CDR3 segments were not shared among different patients [41,57–63,74–

78,80,81]. There is extensive promiscuity in the interactions of TCR with peptide/MHC and a number of reasons may be responsible (reviewed in [80]): (i) Different T-cell clones expressing different TCR recognize peptides bound to different MHC class I or II alleles. Different peptides from the same antigens may be presented to T cells; (ii) Different antigenic peptides bind to the same MHC allele and these different peptide:MHC complexes may be recognized by different TCR; (iii) Different epitopes of a single peptide:MHC complex may be recognized by different TCR; (iv) The same peptide:MHC epitope may be recognized by several different TCR; (v) Several amino acids of the CDR3 are TCR clone specific and may be coded, at least in part, by random additions of N-nucleotide (nontemplated nucleotides) during the generation of T-cell diversity and not by nucleotides belonging to V, D (β -chain only) or J gene segments [74]. However, a substantial number of CDR3 amino acid motifs, comprised of two amino acids each, were expressed in higher proportions (statistically significant) in the CDR3 of several T-cell clones from AAA lesions in a number of patients vs. those of PBMC from normal donors, used as methodological controls (Table 5). These CDR3 two amino acid motifs, may be coded by nontemplated nucleotides, (see above, [74]), and may have undergone selection by antigenic stimulation. Preferential V β 22 and V β 25 utilization was found [83] in aneurysmic lesions from 10 of 14 patients with Marfan Syndrome, familial thoracic aortic aneurysms (TAAs) and patients with sporadic TAAs.

We studied here TCR transcripts from fresh (uncultured) T cells, and not T cells expanded *in vitro*, in culture with recombinant IL-2 (rIL-2). T-cell lines expanded in culture with rIL-2 are comprised of different proportions of T-cell clones vs. those present in fresh, uncultured, T cells from the same donor, and exhibit different properties such as cytokine production [84]. Growth rates of different T-cell clones in culture with rIL-2 are often different. Expansion of T cells in different concentrations of rIL-2 yields T-cell lines with quite different properties [85].

The $\alpha\beta$ TCR+ T-cell repertoire is very large [44–46,62,63] and the maximum theoretical number is 1018 different α/β TCR, 107 α -chain, and 1012 β -chain TCR transcripts [62]. Each T-cell clone is identified by a unique TCR and recognizes a different antigenic epitope (peptide plus MHC) through its TCR, which is the unique fingerprint of that particular T-cell clone. The number of T-cell clones is greatly reduced during thymic selection and only a small proportion survive and become mature T cells. Arstila et al [44] estimated that 1×10^6 different β -chain TCR transcripts may be expressed in different T-cell clones in PBMC of normal donors. Each one of them may be pairing with 25 or more different α -chain TCR [44]. Warren et al [63] used high-throughput sequencing of β -chain TCR to measure at least 1×10^6 distinct β -chain TCR in PBMC. Robins et al [45] used deep sequencing and a Poisson statistical model to estimate $3\text{--}4 \times 10^6$ β -chain TCR sequences in the peripheral blood, a 4-fold higher than the other two estimates. Qi et al [46] employed next-generation sequencing and non-parametric statistics to estimate the size of the β -chain TCR repertoire to be 100×10^6 β -chain TCR sequences in the peripheral blood. The size of the T-cell repertoire is very large and the number of different T-cell clones is very high and able to recognize all conceivable antigenic epitopes. Therefore, the probability is very small of finding by chance two or multiple identical α - or β -chain TCR transcripts in an independent sample of T cells. The presence of multiple identical copies of α - or β -chain TCR transcripts has to be the result of specific antigen-driven proliferation and clonal expansion of particular T-cell clone(s) *in vivo* in AAA lesions, in response to as yet unidentified antigen(s), self or non-self, that they recognize.

Several lines of evidence (Table 1), strongly suggest that autoimmunity may be responsible for the pathogenesis of AAA, which may be an autoimmune disease [3–5, 9–41]. The identification of the three components of the trimolecular complex in AAA, and in particular the clonal expansions of the α - and β -chain TCR of T cells in AAA lesions ([10, 41], and this study), the association of AAA with MHC Class I and II [20–22] and the identification of

putative AAA antigens [23–36], provide a compelling argument that AAA is a specific antigen-driven T-cell disease. AAA formation is controlled by cells, cytokines, and small molecules that inhibit inflammation (Table 1, [86]). Impaired immunoregulation may also play a role [38–40]. Chronic inflammation mechanisms in AAA are typical to those in autoimmune disease [87] and the immune response to tumors [88]. However, formation of transient follicles has been observed during the destabilization of atherosclerotic plaques [89] and it could be suggested that such a mechanism may be responsible for the presence of mononuclear cell infiltrates in AAA lesions and that the immune response identified in AAA may be secondary in the disease process. However, others emphasize the differences between AAA and atherosclerosis and the increasingly popular view of the autoimmune hypothesis as responsible for the etiology of AAA versus the atherogenic theory [90]. Nevertheless, our understanding of the pathogenesis of AAA is still limited.

AAA is associated with certain HLA class I (HLA-A2, HLA-B61) and class II (HLA-DerRB1*02, -DRB1*04) alleles [20–22,35]. Three (AAA03, AAA09 and AAA10) of the 5 patients studied here were typed by DNA-based HLA-typing approaches and had DRB1 alleles positive for the DRβGln70 amino acid residue (Table 4). These 3 patients exhibited in AAA lesions statistically significant clonal expansions of both α- and β-chain TCR transcripts. DNA-based HLA-typing of 6 patients with AAA [41], including the 3 patients shown here, revealed the expression of the DRβGln70 amino acid residue in 5 of 6 patients [41]. Clonally expanded β-chain TCR transcripts were found in 5 of these 6 patients and 4 expressed DRβGln70 [41]. The DRβGln70 amino acid residue is associated with AAA [21, 22] and it forms together with amino acid residues in positions 67, 71 and 74 a binding peptide pocket (#4) in HLA-DRB1 [91,92], which is associated with certain autoimmune disorders [91–93]. A large number of DRB1 alleles with Gln at position 70 of the β-chain have been identified [55].

Our studies demonstrate clonally expanded αβ TCR in AAA lesions and may permit identification of the three molecular components of the trimolecular complex, the αβ TCR, the HLA-DRβGln70 (and perhaps other HLA epitopes) and the AAA-associated antigens (peptides), self [3,18,19,23–26,30] and non-self [31–36], responsible for the immunopathogenesis of the disease. These antigens may be involved in the immunopathogenesis of AAA and in particular the initiation and/or the propagation of the disease. Molecular mimicry [37], which is defined as the sharing of cross-reactive antigenic determinants between host antigens and microorganisms, including viruses or bacteria, may be involved in the pathogenesis of AAA [35]. AAA may be initiated by an immune response to a virus or bacterium, which may cross-react with an antigenic epitope of a self-antigen, by molecular mimicry. After the clearance of the microorganism, the initial immune response may be propagated by the crossreactive antigenic epitope(s) of a self-antigen [37]. Molecular mimicry may be more likely responsible for the pathogenesis of the disease than purely autoreactive T cell clones. T cells with high affinity for self-antigenic determinants would have been eliminated during thymic selection. In contrast, T cell clones that recognize crossreactive antigenic determinants of host antigens and microorganisms may escape elimination during thymic selection. In addition to initiation, propagation/progression is very important for the development of clinical disease. The evidence suggesting that the immune system is responsible for propagation/progression of the disease is strong and it is further supported by the unique ability of the immune system to exhibit immunological memory.

Our results (this report and [10,41]) provide strong evidence supporting the hypothesis that AAA is a specific antigen-driven T cell disease. The identification of the clonally-expanded TCR transcripts in AAA lesions, may permit the identification of the antigens, self or nonself, recognized by the clonally expanded T lymphocytes. These AAA-associated antigens may play a critical role in the initiation and/or the propagation of the disease, and identification of their

role is critical for understanding AAA and may permit the development of new therapies for the management of aneurismal disease.

Supporting information

S1 Table. Human α -chain TCR primers used for amplification.

(DOCX)

S2 Table. Additional α -chain TCR Transcripts (CDR3 Region) to those shown in Table 4, Expressed in the Aneurysmal Wall of Patient AAA03. These α -chain TCR transcripts were unique when compared to each other.

(DOCX)

S3 Table. Additional α -chain TCR Transcripts (CDR3 Region) to those shown in Table 4, Expressed in the Aneurysmal Wall of Patient AAA09. These α -chain TCR transcripts were unique when compared to each other.

(DOCX)

S4 Table. Additional α -chain TCR Transcripts (CDR3 Region) to those shown in Table 4, Expressed in the Aneurysmal Wall of Patient AAA10. These α -chain TCR transcripts were unique when compared to each other.

(DOCX)

S5 Table. Additional α -chain TCR Transcripts (CDR3 Region) to those shown in Table 4, Expressed in the Aneurysmal Wall of Patient AAA12. These α -chain TCR transcripts were unique when compared to each other.

(DOCX)

S6 Table. Additional α -chain TCR Transcripts (CDR3 Region) to those shown in Table 5, Identified in PBMC from Normal Donors. These α -chain TCR transcripts were unique when compared to each other.

(DOCX)

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