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Circulating Activated and Effector Memory T Cells Are Associated with Calcification and Clonal Expansions in Bicuspid and Tricuspid Valves of Calcific Aortic Stenosis

Robert Winchester,* Margrit Wiesendanger,* Will O’Brien,* Hui-Zhu Zhang,* Mathew S. Maurer,† Linda D. Gillam,† Allan Schwartz,† Charles Marboe,‡ and Allan S. Stewart§

We sought to delineate further the immunological significance of T lymphocytes infiltrating the valve leaflets in calcific aortic stenosis (CAS) and determine whether there were associated alterations in circulating T cells. Using clonotypic TCR β-chain length and sequence analysis we confirmed that the repertoire of tricuspid CAS valves contains numerous expanded T cell clones with varying degrees of additional polyclonality, which was greatest in cases with severe calcification. We now report a similar proportion of clonal expansions in the much younger bicuspid valve CAS cases. Peripheral blood flow cytometry revealed elevations in HLA-DR⁺ activated CD8 cells and in the CD8⁺CD28nullCD57⁺ memory-effector subset that were significantly greater in both bicuspid and tricuspid CAS cases with more severe valve calcification. Lesser increases of CD4⁺CD28null T cells were identified, principally in cases with concurrent atherosclerotic disease. Upon immunostaining the CD8 T cells in all valves were mainly CD8⁺CD28null, directly supporting the relationship between immunologic events in the blood and the valve. The results suggest that an ongoing systemic adaptive immune response is occurring in cases with bicuspid and tricuspid CAS, involving circulating CD8 T cell activation, clonal expansion, and differentiation to a memory-effector phenotype, with trafficking of T cells in expanded clones between blood and the valve. The Journal of Immunology, 2011, 187: 1006–1014.

Calcific aortic stenosis (CAS) is characterized by progressive remodeling of the valve matrix, which involves the formation of calcific nodules. The thickened and less compliant valve leaflets narrow the valve aperture, resulting in left ventricular hypertrophy and ultimately heart failure (1). CAS is often relentlessly progressive and valve replacement is currently the only treatment. It has been classically envisioned as a degenerative response of the aortic valve to hemodynamic stress as reflected in its designation as senile aortic stenosis (2). CAS is relatively frequent, developing in 2–3% of the population by the age of 75. However, nearly 10% of those with the uncommon and hemodynamically less adequate anatomic variant bicuspid valve develop CAS two or more decades earlier than do those with tricuspid valves.

The normal valve leaflet is avascular and without evidence of infiltrating mononuclear cells. The more recent identification of a prominent mononuclear cell infiltrate in the vast majority of both bicuspid and tricuspid CAS valves introduced the concept of inflammation to this disease (3–7). The infiltrate predominantly consisted of variably sized aggregates of activated T cells and macrophages adjacent to regions of heterotopic calcification and neoangiogenesis. However, the questions of the immunologic nature of the T cell infiltrate in the valve and whether it was part of a broader systemic immune response remained unanswered. In particular, it was not known whether the infiltrate entirely consisted of numerous polyclonal T cells that may be entering the valve secondarily in response to calcification and injury, or whether the infiltrate contained expanded T cell clones, which would suggest the possibility that T cells might play a more important pathogenic role in aspects of CAS through their activation, differentiation, clonal expansion, and mediator release in an adaptive immune response.

This question was addressed in an initial study of the TCR β-chain length and sequence analysis in the repertoire of the T cells infiltrating tricuspid CAS valves where on average more than half of the infiltrating oβ T cell repertoire was shown to consist of expanded clones (8). Additionally, the large majority of expanded clones found in either blood or the valve exhibited clonotypes unique to that compartment and were not shared, emphasizing that the clonal expansion in the tricuspid valve was immunologically specific, although whether β-chain sequence analysis would show that clonal expansions were similarly present in bicuspid valve CAS was not determined. In contrast, normal valves did not have identifiable TCR β-chain transcripts (8).
These findings of clonal expansions in the valves of CAS cases along with evidence suggesting involvement of CD8 T cells raised the major question of whether the T cells in the circulation of those developing CAS would exhibit features of an ongoing systemic immune response and furthermore whether the response would be greater in the CD8 subset. The alteration in T cell phenotype during an immune response, especially evident in the CD8 T cells, usually includes the transient expression of activation molecules, such as HLA-DR (9), and the development of memory-effector cells particularly in sustained responses, defined by the loss of costimulatory CD28 molecules and acquisition of structures including NK receptors, for example, CD57 (10–13).

Accordingly, we hypothesized first, that the entrance of T cells into the valve might be a reflection of a major systemic immune response that would be characterized by T cell activation and differentiation to memory-effector phenotype in the blood; second, that the intensity of these systemic immune events would be related to the severity of the changes of CAS; and third, that clones shared between blood and the valve, reflecting lymphocyte trafficking, would be found in the memory-effector CD8*CD28null T cell subset.

However, tricuspid valve CAS is a disease of the elderly, and in normal chronologic aging the prevalence of CD28nullCD57+ T cells increases in the CD8 subset (8). The alteration in T cell phenotype in the blood, reflecting lymphocyte trafficking, would be found in the memory-effector CD8*CD28null T cell subset.

The severity of aortic valve pathology in CAS was quantified after Warren study followed institutional guidelines and was approved by the Columbia University Institutional Review Board. Cardiac valves and venous blood samples were obtained from 18 tricuspid and 9 bicuspid CAS cases with advanced CAS and/or ascending aortic disease at the time of valve replacement surgery. The B or T suffix of the CAS case identifier refers to the bicuspid or tricuspid valve structure. All of the study subjects participated voluntarily and gave informed consent. The study followed institutional guidelines and was approved by the Columbia University Institutional Review Board.

**Pathologic grading of calcification**

The severity of aortic valve pathology in CAS was quantified after Warren and Yong (18). Atherosclerosis was defined either as the requirement for a revascularization procedure or as asymptomatic aortic or coronary artery atherosclerosis directly observed during surgery. Those with extensive histories of multiple (two or more) atherosclerotic events (e.g., peripheral vascular, coronary, carotid) are designated “V” (vasculopathic, n = 6). In one case the event requiring revascularization was 3 mo prior to aortic valve replacement, whereas in the others it was remote and ranged up to 30 y prior to valve surgery. Stable mild coronary artery disease for ≥1 y prior to valve replacement surgery, or those without clinical evidence of the presence or consequence of atherosclerotic disease, but who were found to have coronary artery or aortic atherosclerotic involvement during surgery, were designated as stable atherosclerotic disease cases, or “S” (n = 8). One additional case designated had polymyalgia rheumatica and an ascending thoracic aortic aneurysm. This case was not considered to have atherosclerotic disease.

**Flow cytometry and immunohistochemistry**

PBMCs were stained with a combination of Abs, including FITC- or allophycocyanin-conjugated anti-CD3, PE- or allophycocyanin-conjugated anti-CD8, PE-conjugated anti-CD28 or anti-CD57 mAbs, or FITC-conjugated anti–HLA-DR Abs (all from BD Biosciences). Additionally, CD56, CD16, CD158c1 (KI3DL1, NK1), and CD69 were used. For the isolation of purified T cells, CD4+ T cells were first separated from blood mononuclear cells by positive selection using magnetic beads (Dynal). In a second step, the CD4-negative fraction was stained with anti-CD28-allophycocyanin and anti–CD28-PE for cell sorting (FACSaria; BD Biosciences).

For double immunostaining, the paraffin sections were stained with the first primary Ab from either rabbit anti-human CD3 or CD4 (Cell Marque, Rocklin, CA) or goat anti-human CD28 (R&D Systems, Minneapolis, MN) or monoclonal mouse anti-human CD8 or CD20 (Dako). This was followed by the addition of a species-specific secondary secondary biotinylated Ab and streptavidin conjugated with Alexa Fluor 555 (Vector Laboratories). After blocking, second stage staining was similarly performed followed by fluorescein-conjugated avidin and DAPI counterstaining. The three fluorescent images were taken at different wavelengths and merged. Four to six images were used to count the proportion of CD8*CD3+ T cells in the samples.

**T cell repertoire β-chain length distribution analysis**

Total RNA from homogenized valve tissue or separated T cell subsets was extracted and reverse-transcribed as described (19). The CD3 region within the TCR β-chain and its flanking sequences were amplified in 24 separate PCRs using forward primers specific for each TCR Vβ family (8). The international ImMunoGeneTics information system and Arden nomenclature for V region families are used (8, 20, 21). Each of the PCR products was examined in TCR β-chain length distribution (spectratype) analysis as performed as described (21) using an ABI 3100 capillary sequencer. The output files containing peak size in base pair, height, and peak area was used to create histograms of peak area versus size and then converted to peak area versus CDRL3 length plots. For each Vβ family, a reference repertoire was compiled from the averaged peripheral CD4+ T cell β-chain length distribution of 15 individuals (22). The Hamming distance (HD), a measure of the difference between a histogram describing the TCR β-chain length distribution of a polyclonal reference repertoire and the test repertoire length distribution, was calculated for each Vβ family as the difference in normalized peak area between the test population and the reference (range, 0–100, with 0 indicating that the test repertoire diversity was maximal and equivalent to the polyclonal reference of CD4 T cells from healthy individuals, and 100 indicating that no repertoire product was shared with the reference repertoire) (8, 23). If a Vβ product was not detected in the sample for a particular family, that result was excluded when the HD for each T cell subset was averaged across all BV families tested, yielding a mean HD (mHD). The proportion of the repertoire occupied by a given BV family was approximated by summing the peak intensities for all spectratype peaks across all BV families and determining the proportion attributable to a given BV family.

**TCR β-chain sequence analysis**

To define the degree of clonal expansion in the circulating T cell subsets and identify clonal trafficking between blood and the valve, limited cloning and nucleotide sequencing of selected amplified β-chain CDR3 regions were performed as described (8). These samples were selected on the basis of the initial spectratype analysis, and they usually included combinations of samples that shared oligoclonal expansions at the same CDR3 length. Typically, 48 clones were sequenced for each BV family for each valve or peripheral blood subset. CDR3 sequences were edited and aligned (Geneious) and analyzed using international ImMunoGeneTics information system/V-QUEST criteria (20). Representative sequences from the valve leaflets and blood subsets comprise GenBank accession numbers JF733129–JF7331232 (http://www.ncbi.nlm.nih.gov/genbank). In the instance of an expanded clone in a given site with multiple identical sequences, only one representative product was shared with the reference repertoire, and the clone size noted. To describe repertoire diversity in each Vβ family analyzed, the Simpson diversity index (SDI) as a measure of the repertoire sequence diversity was calculated as described (24). The SDI ranges from 0 to 1, with 1 representing maximal diversity (a polyclonal repertoire consisting of unexpanded T cells) and 0 representing maximal contraction (a single monoclonal expansion). The clonotype frequency in a given BV family was calculated from the number of sequences in the given clone divided by the total number of sequences obtained for that BV family in the particular sample. The proportional representation of each BV family in the repertoire was approximated by summing the peak intensities for all peaks across all BV families and determining the fraction accounted for by a specific BV family. This latter calculation is potentially influenced by differences in PCR efficiencies and is considered an approximation. The raw number of sequences across the entire repertoire in the sample occupied by the expanded clone in a sample across the entire repertoire was reported as the product of the clonotype frequency in a given BV family times the proportional representation of that BV sample.
Statistical analysis

The Wilcoxon rank-sum test was used for comparisons of valve calcification score, chronicologic age, aortic valve subtype, and immunophenotype. A Spearman or nonparametric correlation (two-tailed) was used to assess the relationship between the relative proportion of T cell subsets and the grade of valve calcification. A t test was used to compare the means of independent samples. A two-tailed p value < 0.05 was considered significant. SPSS 17 software was used for all analyses (SPSS, Chicago, IL).

Results

Peripheral blood T lymphocytes express activation markers in CAS

The proportion of circulating CD3+ T cells expressing HLA-DR in CAS was considerably increased in the peripheral T cell compartment (range, 4.7–32.9%; mean, 16.1 and 16.3% in tricuspid and bicuspid CAS, respectively) compared with the expected frequency of ≤5% in healthy controls (Table I). HLA-DR expression on T cells was more strikingly increased within the CD8 subset (mean, 24.4 and 28.4% in tricuspid and bicuspid disease, respectively; range, 5.6–42.8%) (Table I). CD69 expression also correlated with HLA-DR expression on T cells (data not shown). A greater proportion of activated HLA-DR+ T cells was observed in those with a calcification score p 0.530, respectively; range, 5.6–42.8%) (Table I). CD69 expression also correlated with HLA-DR expression on T cells (data not shown). A greater proportion of activated HLA-DR+ T cells was observed in those with a calcification score p 0.03; correlation, p = 0.530, p = 0.024). Interestingly, the percentage of the CD8+CD57+ T cell subset expressing HLA-DR ranged up to 49.9% and was greater than that found on CD8 T cells in 11 of 14 CAS cases, indicating that the CD8 T cells that have differentiated to a memory-effector phenotype continue to be strongly activated (Table I). This increase in HLA-DR and CD69 expression was independent of the atherosclerosis status of the cases. Additionally, the expression of HLA-DR on CD4 T cells was increased over the reference samples in 10 of 18 cases (mean, 6.9%) but was considerably less marked than that observed on CD8 T cells (Table I).

Peripheral blood of CAS cases contains an expanded population of CD28+ memory-effector T lymphocytes

The proportion of circulating CD8+ T cells that extinguished expression of CD28, indicating differentiation to the memory-effector phenotype, was substantially increased in tricuspid CAS (range, 36.6–96%; mean, 69.7% of CD8 T cells) and in bicuspid CAS (range, 20–85.5%; mean, 65.8%) (Table I). Among tricuspid cases, the percentage of CD8+CD28+ T cells and calcification remained significant even when controlling for age (p = 0.694, p = 0.003). Although the mean age of patients was 79 ± 8 y, the correlation between CD8+CD28+ T cells and calcification score was p = 0.590 (p = 0.001). Further phenotyping, to verify that the CD8 T cells had additional characteristics of the effector memory subset (25), showed that the frequency of CD8+CD57+ T cells was elevated (mean, 47 and 48.4% in tricuspid and bicuspid CAS, respectively) (Table I). We also measured the per cell expression of CD56, CD16, and CD158c1 (KIR3DL1, NKBI) on CD4 and CD8 T cell subsets and these structures were also principally expressed on CD28+ T cells (data not shown).

Table I. Among circulating T cells in CAS, the CD8 lineage exhibits the greatest differentiation to an activated and memory-effector phenotype

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ID case number prefixes T and B refer to tricuspid or bicuspid valves, respectively. The CAS score is a measure of severity of pathologic changes. 1 IDs included patients who were clinically stable atherosclerosis present as temporally remote need for a single revascularization procedure, or asymptomatic coronary artery or aortic atherosclerosis found at time of surgery; V, vasculopathic with history of multiple occlusions requiring revascularization procedures.

For atherosclerosis or associated comorbidity, the following terms apply: 0, not detectable; PMR, polymyalgia rheumatic and dissecting ascending aortic aneurysm; S, clinically stable atherosclerosis present as temporally remote need for a single revascularization procedure, or asymptomatic coronary artery or aortic atherosclerosis found at time of surgery; V, vasculopathic with history of multiple occlusions requiring revascularization procedures.
In peripheral blood, the percentage of CD4+CD28null T cells was more moderately elevated (mean, 14.8%; range, 2.6–50.5%) (Table I). This subset was increased among cases with extensive valvular calcification (CAS score ≥ 4; p = 0.007). There was a significant difference in mean frequency of the CD4+CD28 T cell subset between the atherosclerotic-positive (19.36%) and -negative (6.94%) subsets (p = 0.007396), whereas all other cell populations in the table did not differ significantly between the two atherosclerotic disease subsets. However, there was no difference between cases with stable isolated atherosclerotic disease requiring a revascularization procedure in the past and extensive ongoing vasculopathic disease. The mean percentage of CD4+CD57+ T cells was 12.7%.

**T cell repertoire in CAS valves**

Fig. 1 summarizes the repertoire characteristics of the infiltrating T cells for each BV family in 16 tricuspid CAS valves in terms of the number of peaks found on the β-chain length distribution and their peak area in fluorescence intensity units, illustrating that despite considerable heterogeneity, most valve-infiltrating T cells in most valves and in most BV families consist of oligoclonal expansions, as shown by entries containing one, two, or three peaks. The average quantity of the BV family was classified as moderate (mean, 14.8%; range, 2.6–50.5%) (Supplemental Table I). Additionally, a median of 13 BV families across all samples had no detectable or very low expression (<20,000 fluorescent units), consistent with highly selective entrance of clones into the valve.

Concerning heterogeneity among CAS samples in the extent of multi- or polyclonality versus oligoclonality, 7 CAS cases had at least one BV family with six or more peaks and 13 valves had fewer than six peaks in all BV families (Fig. 1). Fig. 1 includes quantification of the estimated T cell input determined by real-time PCR, which is represented in terms of the RNA in the given number of peripheral blood T cells; however, because of variability in per cell mRNA in tissue, this does not represent the actual number of T cells in the tissue sample. No polyclonal BV families were found in any of the cases with CAS calcification scores of 1 or 2. Increasing valve polyclonality, as measured by lower mHD, correlated with estimated T cell input rank order, reflecting recovery of greater numbers of T cells from the polyclonal valves (p = −0.525, p = 0.039).

In eight valves selected to represent the observed range of calcification severity, the presence of individual clonal expansions in the TCR repertoire was confirmed with higher resolution β-chain nucleotide sequencing (Supplemental Table I). Some clones were considerably expanded, and when adjusted for the proportion of the BV family representation, they were calculated to occupy a considerable fraction of the repertoire of valve-infiltrating T cells. For example, clone JF731133 was calculated to occupy 10.94% of the repertoire of valve-infiltrating T cells (Supplemental Table I). The sequence data summarized in Table II using an SDI of 0.8 further emphasize the departure of the valve TCR repertoire from a polyclonal distribution. Although there was no correlation between β-chain length polyclonality, as measured by mHD, and CAS score, the degree of TCR repertoire polyclonality in the valve as measured using β-chain nucleotide sequencing data (Table II) was directly and significantly correlated with the severity of calcification (p = 0.887, p = 0.003).

**Immunohistologic staining of valves**

Immunostaining of the valves was performed to define the phenotype and distribution of infiltrating T cells in relationship to the repertoire analyses. Valves with multi- or polyclonal T cell infiltration usually exhibited many regions of very abundant infiltration by CD8 and CD4 T cells (Fig. 2A, 2B). This abundance on staining was consistent with estimated T cell recovery from the valve during RNA extraction (Fig. 1). Sometimes infiltration by CD20 staining B cells was also found in these valves (not illustrated). In contrast, more oligoclonal samples (Fig. 2C, 2D) had more sparse regions of T cell infiltration that predominantly stained for CD8. Among the 14 cases available for immunostaining, the percentage of CD3 T cells that expressed CD8 directly correlated with the degree of repertoire oligoclonality as measured by mHD (p = 0.679, p = 0.011) (Table II). In all valves, most CD8 T cells either lacked detectable ex-
Expression of CD28 or staining was very dim, as shown in Fig. 2B and 2D. These CD28null or CD28dimCD8+ T cells were often found in proximity to sites of calcification (not illustrated) or neovascularization (Fig. 2C). Reciprocally, in valves with greater proportions of polyclonal T cells, with higher percentages of CD4+CD3+ T cells, more were CD28nullCD8+.

Clonal expansions in circulating T cell subsets

Three examples of the spectratypes of the circulating T cell subsets and those of the paired valve are illustrated in Fig. 3. Comparison of Fig. 3B with Fig. 4 shows that in general the overall results of the low-resolution spectratype method parallel the distribution of clone β-chain sequences. A marked degree of oligoclonality in the circulating CD8null CD8+ memory-effector subset was seen and it was usually more restricted than the heart valve. A summary analysis of the clonal composition of the TCR repertoires in two subsets of circulating CD8 T cells also showed that the CD8+CD28null repertoire has the greatest degree of oligoclonality (mHD, 61.1) (Table II). This oligoclonality was also illustrated by sequenc- ing in representative case T54 (Fig. 4, Table III, Supplemental Table I). In contrast, the CD4+ repertoire was uniformly highly polyclonal (Figs. 3, 4, bottom right panel) (mHD, 19.5; SDI, 1.0) (Table II). Fig. 4 shows that the CASSLDT sequence (JF731330) accounts for virtually all of the TRBV7 memory-effector repertoire (97.4%) and that this clonotype accounts for 2.47% of this individual’s entire CD8 T cell repertoire, as shown in the Supplemental Table I. Interestingly, the CD8+CD28+ T cell subset in CAS also contained a variable number of clones that have undergone limited proliferation (mHD, 43; SDI, 0.8) (Table II, Figs. 3, 4) (e.g., JF731135, JF731137, JF731139, and JF731222–JF731229). Table III illustrates three examples of the clonotype frequency of selected clones in the CD8+CD28null and the CD8+CD28+ T cell subset, showing that the clonal expansions in these instances occupied up to 5.41% of the total subset repertoire in the case of the CD8+CD28+ T cell subset and 1.51% of the CD8+CD28null repertoire.

Identification of the same expanded clones in both valve tissue and peripheral blood

Of 33 expanded clones in the peripheral blood found by sequence analysis, 27 clonotypes were not present in the valve, and 13 of 19 clones expanded in the valve were not detected in blood, indicating that T cell entrance into the valve was selective and expansion may have occurred in the valve.

### Materials and Methods

For the SDI, an index of repertoire diversity determined from β-chain nucleotide sequencing, 1.0 indicates polyclonal. For the mHD, the average of the distance between the observed TCR β-chain length distribution and a polyclonal reference, 0 indicates polyclonal.

### Table II. Oligoclonality in the T cell repertoire of CAS valves and in peripheral blood phenotypic subsets, determined by β-chain nucleotide sequencing and by β-chain length distribution analysis

<table>
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<th>ID</th>
<th>CAS Score</th>
<th>SDI</th>
<th>mHD</th>
<th>CD8 %a</th>
<th>% CD8 T Cells</th>
<th>CD8%CD28null</th>
<th>CD8%CD28+</th>
<th>CD4+</th>
<th>Repertoire Complexity</th>
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ID case number prefixes T and B refer to tricuspid or bicuspid valves, respectively. The CAS score, which measures the severity of pathologic changes, is defined in Materials and Methods. For the SDI, an index of repertoire diversity determined from β-chain nucleotide sequencing, 1.0 indicates polyclonal. For the mHD, the average of the distance between the observed TCR β-chain length distribution and a polyclonal reference, 0 indicates polyclonal.

aCD8 refers to percentage of CD3+ T cells in the valve that are CD8+ by double immunostaining (data not shown for seven additional samples).

—, no data.
occur after entrance of a progenitor T cell (Supplemental Table I and data not shown).

Fig. 3 depicts the spectratype result showing the striking predominance of an expansion at CDR3 length 12 in the first three frames. The subsequent sequence-based repertoire analysis of this sample in Table III illustrates that this peak consists of a single shared CD8 T cell clonotype. The CD4 T cell repertoire was polyclonal.

**Table III** shows that in sequence analysis a clone, JF731133, was present in the valve in case T55 with the CDR3 sequence CASSLALAFNEQFF and length 12, and that this accounted for 85.7% of the TRBV11 repertoire. Moreover, the sequence of this clone was identical, including regions encoded by N and P diversity nucleotides, to the sequences of clones present both in the circulating CD28nullCD8+ subset, JF731134, and the CD28nullCD8+ memory-effector subset, JF731135, which account for 17.7 and 20% respectively, of the two CD8 subset repertoires (Table III). The JF731133 clone is substantially expanded in the valve, accounting for 10.94% of the entire repertoire of T cells infiltrating the valve. The presence of the same clonotype in these samples suggests clonal traffic between the circulation and the valve.

**Discussion**

In this study we sought to evaluate the immunological significance of T lymphocytes infiltrating the valve leaflets in CAS. The central findings included, first, that clonal expansions in cases with bicuspid or tricuspid CAS valves were associated with parallel changes in the circulating lymphocyte population, including marked T cell activation, denoted by HLA-DR expression, and extensive clonal expansion with differentiation to memory-effector status, reflected by extinguishing expression of CD28 and acquisition of NK markers. These changes were most strikingly evident in the CD8 T cell subset and were highly correlated with the
severity of valve calcification and stenosis. Second, upon immuno-
staining of the valve, the infiltrating CD8 T cells were mainly
CD28null or CD28dim. Third, we extended the initial finding in
tricuspid CAS (8) by identifying similar clonal expansions in bi-
cuspid valves by β-chain sequencing and obtaining evidence in-
dicating that polyclonal CD4 T cells often also infiltrated valves
with higher degrees of calcification. Lastly, the clonal expansions
in the valve were directly linked to the activation and differenti-
ation events in the peripheral blood by demonstrating trafficking
of members of the same T cell clone between the peripheral cir-
culation and the valve. Taken together, our data suggest the in-
terpretation that a systemic adaptive immune response is occurring
in individuals developing CAS, and that this systemic response is
coupled to the lymphocytic infiltration of the valves, where it
could be driven by the recognition of peptide Ags expressed in the
valve.

Several findings suggest an important role of CD8 T cells and, in
particular, the CD8+CD28null T cells in CAS. They include iden-
tification of this subset in the valve on immunostaining, as well
as the correlation of the degree of its expansion in blood with
the severity of valve calcification. Activation and differentiation
to memory-effector status among circulating lymphocytes also
strongly predominated in the CD8 T cell subset. Moreover, HLA-
DR expression was particularly increased in the CD8+CD57+
T cell subset, indicating their continued activation. Furthermore,
eight instances of sharing of the same T cell clones expanded both

Table III. Two examples of shared T cell clones identified in the aortic valve and in peripheral blood phenotypic subsets with identical
β-chain sequences, indicating clonal trafficking

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Case</th>
<th>Sample Source</th>
<th>CDR3 Region Nucleotide Sequence, Junction Analysis, and Translationa</th>
<th>No. of Sequences</th>
<th>Clonotype Frequency in Repertoire (%), β-chain</th>
<th>Clonotype Frequency in Blood (β-chain), β-chain</th>
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<td>T55</td>
<td>Circulating CD8+CD28neg T cells</td>
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<td>TGTGCCAGCAGCTTAGcttt</td>
<td>11</td>
<td>55</td>
<td>5.41</td>
</tr>
</tbody>
</table>

aUppercase denotes germ line residues encoded respectively by the TRBV element, TRBD element (underlined), and TRBJ element. Lowercase denotes N or P nongermline-encoded nucleotides.

bOf the number of identical TCRB sequences found in the sample that comprises the expanded clone, only one sequence has been entered in GenBank.
in the valve and in the peripheral blood memory-effector CD8\textsuperscript* CD28null T cells shown in Fig. 4 and Table III directly link clonal entrance and expansion of T cells within the valve to the activated and memory-effector T cells in the circulation. The quantitative characteristics of the expansion of the CD8 T cell clones involved in trafficking between the circulating T cell population and the heart valve included an example of where a shared clone accounted for 10.94% of all of the T cells infiltrating the valve. Additionally, most of these cells had an approximate individual clone frequency in blood of 1–2% or more of all T cells in the CD8 memory-effector subset, suggesting that they account for a considerable proportion of the circulating T cells exhibiting a memory-effector phenotype. Together with the earlier findings where 23 of 24 clones identified both among the circulating lymphocytes and in the valve were CD8 lineage T cells (8), these findings also suggest that the peptides driving this aspect of the immune response in CAS likely have a cytoplasmic origin.

Evidence was also obtained that CD4 T cells might also be involved in aspects of CAS. Maturation to memory-effector subsets of the circulating CD4 T cells was found, albeit at a much lower and more variable level. There were two features to the elevation in CD4\textsuperscript*CD28null T cells: first, the intensity of this response correlated with the severity of valve disease; and second, of great interest, the primary association of elevations in this CD4\textsuperscript*CD28null T cell subset appeared to be with clinically significant atherosclerotic disease (Table I). The relationship between this CD4 T cell subset and unstable atherosclerotic disease and with rheumatoid arthritis is well recognized (26, 27), although none of these conditions was noted in the present CAS cases. In the present study, the levels of this subset did not differ between those with extensive vasculopathic atherosclerosis or stable atherosclerosis. One instance of a shared, but not detectably expanded, clone between the CD4 blood subset was found (Fig. 4), JF731140 and JF731141 in a case that also had atherosclerotic disease. It is possible that this instance of a shared T cell clone reflects the contribution of the comorbid atherosclerotic process (28, 29). To the extent that CD4\textsuperscript*CD28null T cells reflect an atherosclerotic process in the subset of about half of the cases with atherosclerosis, it appears that this process is linked to enhancing CAS severity. Importantly, however, as emphasized by Wu et al. (8), the overall oligoclonal nature and CD8 lineage of valve-infiltrating T cells that predominate in CAS contrast sharply with the findings reported in atherosclerosis (28, 29), emphasizing the difference of the immunopathologic events that characterize CAS.

While oligoclonal expansions were identified in all valves, some valves with higher degrees of calcification also exhibited an additional component of polyclonal T cell infiltration. They had a lower proportion of CD3\textsuperscript*CD8\textsuperscript* T cells and increased CD28null T cells, frequently together with infiltrating CD20\textsuperscript* B lymphocytes. We interpret this to indicate that in valves with a multi- or polyclonal TCR repertoire, more of the infiltrating T cells are naive, clonally unexpanded CD4 lineage cells, which likely do not participate in the process via cognitive recognition by their clonotypic TCR but may reflect a response to chemokines released in later stages of immune-mediated tissue injury. Reciprocally, these findings also suggest that the T cell infiltration initially consists of a relatively few dominant CD8 T cell clonal expansions.

In normal chronic aging the prevalence of CD28nullCD57null T cells increases in the CD8 and, to a much lesser extent, in the CD4 compartment (14) along with large clonal expansions in this subset (15–17). These expansions have been attributed to the sustained proliferation of CD8 T cells involved in maintaining viral latency, as well as the involvement of CD4 and CD8 T cells in diverse chronic inflammatory conditions. It is well recognized that responses to persistent viral infection such as CMV also contribute to the expansion of the CD8CD28null T cell population (30, 31). Five of a subset of 11 cases in Table I examined for the presence of CMV infection by PCR were positive, a level consistent with the age and ethnicity of these cases (32). There was no correlation between CMV positive or negative status and the size of the CD8CD28null population, or the mean CAS score (data not shown). Effros et al. (14) advanced the concept that CD8CD28null T cells are immunosenescent and that their expansion modulates both immune and nonimmune functions and contributes to various age-related pathologies. Intriguingly, the present findings in tricuspid CAS are generally consistent with the association noted by Effros et al., especially given the advanced age of the tricuspid CAS cases, and in this respect tricuspid valve CAS can be added to conditions characterized by expansions of memory-effector T cells. However, our interpretation of the mechanism differs from that proposed by Effros et al. in that CAS does not appear to be a secondary consequence of immunosenescence. Our data are more consistent with the possibility that a specific immune response involving the valve drives the activation of CD8 T cells and their maturation to CD28null phenotype. This interpretation is supported by: 1) the high expression of HLA-DR and CD69 on CD8CD28null T cells in CAS that distinguishes this condition from the CD8CD28null T cell populations in aging; 2) the extent of the expansion of the CD28nullCD8null subset was directly and significantly correlated with the CAS severity score (p = 0.003), but was independent of chronologic age; and 3) perhaps most consequentially, the complete resemblance of the findings in blood and in the valve between the much younger bicuspid valve CAS cases and the older tricuspid valve cases. The counter possibility could be raised that those with bicuspid aortic valve preferentially develop age-inappropriate immunosenescence to account for this finding, but then one would have to propose an explanation for the age-inappropriate immunosenescence. Accordingly, while multiple conditions contribute to the expansion of the CD28null CD8 subset, a major proportion of the elevation in these CAS cases appears to be contributed by the immune processes operating in CAS. However, the study was limited by its overall design and the lack of a large control population of age-matched individuals with varying comorbidities to provide a better measure of the specificity of the findings for CAS.

The questions arise of what immune recognition event, such as a response to intracellular pathogens or self-Ags, might be driving the immune events and what role this immune response plays in CAS. Because CAS occurs in the setting of different clinical situations of bicuspid and tricuspid aortic valves and comorbidities, including hypertension and atherosclerosis that have in common enhanced hemodynamic strain and diminished valvular compliance, we hypothesize that valve mesenchymal cells alter their transcriptional phenotype in response to enhanced strain, resulting in the cellular expression of stress-induced molecules. We envision that peptides from these stress-induced molecules, to which the individual may not be fully tolerated, are expressed in the context of class I MHC, and that an idiosyncratic CD8 T cell immune response is induced in a small subset of individuals. This response seen in the circulating T cells is analogous to a peptide-driven immune response and results in infiltration of the valve by expanding and activated T cell clones that recognize the stress neoantigen. We speculate that these proliferating T cells release cytokines that alter the expression of valve mesenchymal cells and recruit additional lymphocytes into the valve, suggesting that at a minimum one consequence of this inflammation is a further and potentially reversible decrease in valvular compliance. Support for this
possibility comes in part from preliminary data in eight cases where significant production of IFN-γ was found in the CD8+ CD28null T cell subset (data not shown) and from the earlier reports identifying HLA-DR expression on valve mesenchymal cells (33). It is possible that the activated memory-effector CD8 T cells directly injure target valve cells, driving the development of heterotopic calcification, with precedent for development of analogous heterotopic calcification in the setting of the autoimmune mune CD8CD28null T cell response in diseases such as psoriatic arthritis or ankylosing spondylitis. Further study of the immunologic specificity and features of these T cells may provide additional insight into the pathogenesis and the nature of the underlying immune recognition events of this increasingly common valvular disease and bear on whether the immune response might be a potential target for immunosuppressive therapy.

Disclosures

The authors have no financial conflicts of interest.

References