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Analysis of T cell repertoire in hepatitis-associated aplastic anemia

Jun Lu, Atanu Basu, J. Joseph Melenhorst, Neal S. Young and Kevin E. Brown*

Hematology Branch
National Heart Lung and Blood Institute
Bethesda, Maryland

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*Corresponding Author:
Kevin E. Brown
Bldg. 10/Rm. 7C218
National institutes of Health
9000 Rockville Pike,
Bethesda MD 20892
Tel: (301) 496-2479
Fax: (301) 496-8396
E-mail: brownk@nhlbi,nih.gov

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Abstract

Hepatitis-associated aplastic anemia (HAA) is a syndrome of bone marrow failure following an acute attack of seronegative hepatitis. Clinical features and liver histology suggest a central role for an immune-mediated mechanism. To characterize the immune response we investigated the T cell repertoire (T cell receptor (TCR) Vβ chain subfamily) of intra-hepatic lymphocytes in HAA patients by TCR spectratyping. In 6/7 HAA liver samples a broad skewing pattern in the 21 Vβ subfamilies tested was observed. In total, 62% ± 18% of HAA spectratypes showed a skewed pattern, similar to 68% ± 18% skewed spectratype patterns in 3 of 4 patients with confirmed viral hepatitis. Additionally, the T cell repertoire had similarly low levels of complexity. In the PBLs of a separate group of HAA patients prior to treatment, 60% ± 15% skewed spectratypes were detected, compared with only 18% ± 8% skewed spectratypes in healthy controls. After successful immunosuppressive treatment an apparent reversion to a 'normal' T cell repertoire with a corresponding significant increase in T cell repertoire complexity was observed in the HAA samples. In conclusion, our data suggest an antigen-driven T cell expansion in HAA, and achievement of a normal T cell repertoire during recovery from HAA.

Introduction

Hepatitis-associated aplastic anemia (HAA), the development of hematopoietic failure with bone marrow hypocellularity within six months of an episode of hepatitis, is not uncommon, with hepatitis preceding the onset of bone marrow failure in 2-5% of aplastic anemia (AA) cases in Europe and the United States.[1] Aplastic anemia is also frequent following orthotopic liver transplantation for non-A, non-B, non-C hepatitis in young patients: 23%-28% of patients transplanted for non-A, non-B non-C hepatitis developed aplastic anemia, compared to < 1% of all liver transplant patients.[2,3] The hepatitis/aplastic anemia syndrome shows a stereotypical pattern; most often affecting young males, the hepatitis generally follows a benign course but the onset of aplastic anemia 2-3 months later can be explosive and is usually fatal if untreated.[4] The presumed infectious cause of the hepatitis is unknown, but most cases are seronegative for known hepatitis viruses, including hepatitis A, B, C, and G (GBV-C).[5-7] We previously reported 10 cases of HAA seen at the NIH who had evidence of lymphocyte activation; 70% responded to immunosuppression with anti-thymocyte globulin and cyclosporine.[8] Apart from case reports, the presence of lymphocyte activation[9,10] and the clinical response to either immunosuppression or bone marrow transplantation,[11] there is little known of the immunopathogenesis of this syndrome.

The time interval between the occurrence of hepatitis and the onset of bone marrow failure suggests that the initial target organ of the immune response is the liver. For both hepatitis B and hepatitis C infection, large numbers of lymphocytes infiltrate the liver at the time of maximal abnormal liver function,[12,13] and T cells are the predominant inflammatory cells infiltrating the liver parenchyma.[14,15] Analysis of the T cell repertoire

in these cases has demonstrated clonal expansions, and conserved features of antigen specificity in many of these expansions are linked to the immunopathogenesis of viral hepatitis.[16-18] Corresponding information concerning the T cell immune response in HAA in liver or blood is unreported.

T cell receptor (TCR) analysis by spectratyping is a very powerful tool to assess the clonal composition of the T cell repertoire in both infectious and immune-mediated diseases.[19-21] The technique depends on the tissue-specific expression of certain TCRs (on T cells) and on the diversity, somatic rearrangements and insertions at the nucleotide level that create TCR diversity. The majority of mature T cells express an αβ TCR, of which the β chain has three different regions that correspond to the three complementary determined regions (CDRs) of immunoglobulin. The first two CDRs are germline-encoded, but the third CDR is the product of somatic rearrangements. During T cell development, the multiple TCRβ genes for the V, D and J segments rearrange with concomitant nucleotide excision and addition at the VD and DJ junctions, creating the third hypervariable CDR. Depending on the combination of segments and transferase activity, CDR3 sequences of varying lengths are produced, and it is these different CDR3 lengths that the spectratype analysis detects.[22]

We have assessed the T cell repertoire in the liver and peripheral blood lymphocytes in HAA patients and compared the pattern of Vβ subfamily expansion between HAA patients and individuals with hepatitis B and/or C infection by spectratyping. Our results show that many different T lymphocyte clones accumulate in the liver of HAA patients, with a similar broad skewing pattern of the T cell repertoire observed in the liver of patients with HAA as well as known viral hepatitis disease. In

addition, we determined the T cell repertoire in peripheral blood lymphocytes before and after immunosuppressive therapies and showed an apparent reversion to a more 'normal' T cell repertoire in HAA patients after successful treatment.

Patients and methods

Patients and control population

HAA was defined as severe bone marrow aplasia within six months of an episode of documented seronegative (non-A, non-B, non-C) hepatitis. Severity was defined as pancytopenia with at least two of the following abnormalities: absolute neutrophil count less than 500/μL, platelet count less than 20,000/μL, and reticulocyte count less than 60,000/μL, in association with bone marrow cellularity less than 30 percent. Hepatitis was defined as an increase in serum transaminases to at least three times the upper limit of normal (normal alanine transaminase 6-41 IU/L, normal aspartate transaminase 9-34 IU/L).[8] Anonymized liver samples were provided by the University of Minnesota Liver Tissue and Distribution System (LTPADS: NIH Contract #N01-DK-9-2310) after Institutional Review Board (IRB) approval. Additional liver samples were obtained from Cleveland, Columbus, Denver, and Miami. All tissues were obtained after informed consent and following the human experimentation guidelines of the US Department of Health and Human Services and NIH. Liver samples were obtained at the time of liver transplantation and diagnoses included 7 HAA, 4 confirmed hepatitis B and/or hepatitis C infection, and 4 biliary atresia as controls. All patients with HAA were negative for HAV, HBV and HCV infection. All liver samples were immediately frozen and stored at –80 ^{o}C or above liquid nitrogen until RNA was extracted.

Peripheral blood samples or follow up samples were not available from the HAA liver patients. However, peripheral blood was obtained from three additional HAA patients at the time of presentation and after immunosuppressive treatment. All patients were enrolled on National Heart, Lung and Blood Institute (NHLBI) IRB-approved protocols, and one of these patients, was previously reported.[8] In addition, lymphocytes were obtained from ten healthy donors (age < 40 years old) who served as normal controls, and three additional healthy volunteers to assess the sensitivity of spectratype analysis. Lymphocytes were isolated from heparinized peripheral blood by Ficoll-Hypaque density centrifugation (ICN Pharmacentical, INC. Costa Mesa, CA), and used immediately or stored at –80 ^{o}C until RNA was extracted.

Cell sorting by flow cytometry (FACS) for Vβ spectratype sensitivity analysis

In order to confirm that we could detect specific Vβ spectratypes in tissue RNA samples, we first examined the sensitivity of our spectratype assay. Three representative Vβ subfamilies, Vβ2, 14, and 22, were randomly selected. Purified PBLs (10^7) were suspended in 50 μL FACS buffer (phosphate-buffered saline plus 0.4% BSA) and incubated on ice with phycoerythrin-conjugated antibody specific for either TCR Vβ2, Vβ14 or Vβ22, (7 μl, 17 μl or 7 μl respectively; Biodesign International, Saco, ME) for 20 to 30 minutes. After incubation, the cells were washed once and resuspended in 500 μl FACS buffer for cell sorting of antibody-positive and -negative cells on a Cytomation MoFlo Cell Sorter. The sorted Vβ2-, 14-, and 22-positive T cells were mixed with their respective Vβ-negative T cells to produce fixed ratios of the specific Vβs in negative cell populations (0, 10, 10^2,10^3,$10^4$and 10^5 Vβ positive cells in 10^6 total cells for each Vβ

subfamily). Total RNA (~1μg) was extracted from cells (10^6) and standard spectratyping performed. The experiments were repeated three times.

Vβ subfamily specific and " run-off " polymerase chain reactions

RNA was extracted from ground frozen liver samples or PBLs (10^6) by direct suspension in RNAStat 60 (TelTest, Friendswood, TX; 0.1g/mL RNAStat 60). RNA was precipitated by centrifugation according to the manufacturer's instructions, redissolved in ~ 20 μL RNase-free distilled water and the concentration calculated by optical density measurement. First strand cDNA was synthesized using 1 μg of total RNA, reverse transcriptase, and oligo-dT at 42 ^{o}C for 50 minutes and in a final volume of 50 μl following manufacturer's instruction (Invitrogen Life Technologies, Gaithersburg, MD).

CDR3 length analysis was conducted by using a modification of the PCR followed by run-off reaction method previously described.[20] First, PCR amplification of cDNA was performed using 22 specific Vβ primers (21 Vβ primers in the liver samples (Vβ18 was not included for technical reasons); 0.5μl cDNA per reaction) for the TCR Vβ-gene families (excluding Vβ10 and Vβ19, which are pseudogenes) and the common Cβ primer (cgg-gct-gct-cct-tga-ggg-gtc-tgc-g)[20] end-labeled with a fluorescence tag (6-FAM). Amplifications were performed with ExTaq buffer (Takara, Tokyo, Japan), 0.2 mM of mixed dNTP, 0.5 mM of each primer, and 0.5 units of ExTaq polymerase (Takara, Tokyo, Japan) in a volume of 20 μL, on a DNA engine machine (PTC-200, MJ Research, Inc., Waltham, MA). The amplification profile was denaturation at 94°C for 1 min, annealing of primers at 60°C for 1 min and extension at 72°C for 4 min for 40 cycles with a final extension step at 72°C for 10 min. Amplified products were electrophoresed

on 1.5% agarose gels and detected by ethidium bromide staining. For the run-off reaction, a second round of amplification (3 cycles) was performed with a single HEX-labeled fluorescent constant primer internal to the Cβ primer (0.1 mM) in a volume of 10 μL. A standard size marker was added (500 ROX, Applied Biosystems) and first round and run-off products were analyzed on a 310 DNA sequencer using 310 GeneScan Software (Applied Biosystems, Foster City, CA).

Analysis of spectratype

Due to the recombination events that occurs during TCR generation, the size of the amplicon varies in length, and in a normal population of T cells, CDR3 length analysis produces 5~10 identifiable peaks spaced by 3 nucleotides, with fluorescence intensity following a 'quasi-Gaussian' distribution.[20,22] Spectratypes were analyzed in three different ways. First, the spectratype pattern was visually assessed. A normal spectratype profile was defined as showing an approximated Gaussian "bell-shaped" distribution, with discrete peaks spaced by three nucleotides. If discrete peaks were observed but did not have the Gaussian profile, the spectratype was classified as skewed; if discrete peaks were not present, it was scored as 'absent'. To obtain an indication of the magnitude of skewing, each spectratype was assessed as either normal, skewed, or absent by three different observers in a "blinded" fashion. Second, spectratypes were scored mathematically, as previously described.[17,23] Evidence of oligoclonal expansion or skewing was assessed by calculating the relative fluorescence intensity (RI) of each peak (RI (%) = 100 × clonal peak area ÷ total peak area). A skewed profile was determined if either: (i) a single peak was observed and the RI of the dominant peak was

> 35% of total peak area; (ii) two dominant peaks were present and each peak's RI was >25% of total peak area; or (iii) the presence of multipeaks with the dominant peaks differing from a Gaussian pattern and the RI of the peaks > 25% of total peak area. Finally, overall complexity within a Vβ subfamily was determined by counting the number of discrete peaks per Vβ subfamily, with each subfamily graded on a score of 0 to 5.[24] Spectratypes containing more than 5 peaks were given a score of 5, and a score of 0 was assigned if no spectratype signal was obtained; spectratypes with 1, 2, 3, or 4 peaks were given a score of 1, 2, 3 or 4 respectively. The overall spectratype complexity score per sample was calculated as the sum of the scores for each subfamily, with a maximum complexity score for any one patient of 110 (22 Vβ×5).

Statistical analysis

The Student t test was used to assess the differences in Vβ skewing or complexity scores in the different groups of patients. The paired Wilcoxon test (normalizing transformation by Log) was used to determine the significance before and after treatment with immunosuppressive treatment. All statistical analysis was performed by using GraphPad InStat software (GraphPad Software Inc., San Diego, CA).

Results

Sensitivity of T cell repertoire spectratyping

A classic Gaussian spectratype was reproducibly obtained with 10^2 Vβ2 or Vβ14 lymphocytes per 10^6 cells, and with 10^3 Vβ22 lymphocytes per 10^6 cells (Figure 1). With less than these proportions of cells, no amplicon was detected by ethidium bromide

staining, and no products of the correct size were seen by GeneScan analysis. Thus the sensitivity of our spectratype analysis for each Vβ was between 100-1000 cells in a 1 μg total RNA sample.

T cell repertoire of intra-hepatic lymphocytes in HAA patients

Liver histology was not available on two of the HAA livers, but in the other five samples hepatic necrosis with a lymphocytic or mononuclear infiltrate was universally reported. CD3 staining was not performed on any of the livers, although one liver was stained for CD43. Despite this, in one of the HAA patients we were unable to detect any Vβ signal with any primers, despite the presence of RNA as indicated by RT-PCR for GAPDH. Histology of this liver was reported as having a mononuclear infiltrate with neutrophils and occasional eosinophils, and from our sensitivity data we would conclude that the T cell infiltrate was below our detection limit. Similarly we did not detect any Vβ amplicons in any of the four biliary atresia samples. In six of seven HAA livers, 15-21 of the 21 Vβ subfamilies analyzed had detectable PCR products by ethidium bromide staining, and production of a spectratype profile on GeneScan analysis, indicating the presence of a T lymphocyte infiltrate in the livers.

A typical spectratype pattern in HAA is shown in Figure 2: many of the Vβs have a highly skewed pattern. In 70% of the spectratypes there was 100 % concordance among all three observers. As a measure of the skewing in the different patient groups, the numbers of skewed Vβ spectratypes as a percentage of all Vβ spectratypes detected were calculated. There were 59.5±16.7 % (mean±1SD) skewed Vβs in the 21 tested Vβ subfamilies, with 4.8±11.7% giving no detectable spectratype in HAA livers (Table 1).

Similar results were obtained if the relative fluorescence intensity (RI) index was used to determine skewed spectratypes (Table 1). As a measure of the polyclonality of the T cell repertoire the complexity of each Vβ spectratype was assessed and summated to give a total complexity score.[24] The complexity score of the spectratypes varied between 56 and 80, with a mean of 68 (Table 1).

Individual Vβ spectratypes were also analyzed to determine if there were certain TCR families skewed in all HAA patients. Although Vβ 1,7,11,12,15,16, 20, and 24 were skewed in >4/6 of the patients, when we further analyzed the length size of the oligoclonal expansions, no peaks of the same size were found in more than half of the patients. Thus, although oligoclonal expansions affected many Vβ families, there did not appear to be any shared Vβ subfamily expansions for all cases, against a common Vβ CDR3 sequence for all HAA.

The spectratype profiles obtained in the livers of patients with HAA were compared with profiles obtained from four patients with viral hepatitis of known etiology (hepatitis B and/or hepatitis C; Figure 3). In one liver sample of a viral hepatitis patient, no signal could be observed for any of the PCR products, despite the presence of GAPDH product, suggesting a very low number of infiltrating lymphocytes. In the other three samples, there were 67% skewed Vβs of the 21 Vβ subfamilies tested, with no signal in 11% ±13% of reactions. No significant difference was shown statistically in the number of skewed Vβ spectratypes between HAA and hepatitis patients ($p > 0.05$); similarly, the complexity score was not significantly different between these patients.

T cell repertoire of peripheral blood lymphocytes (PBLs) in HAA patients before and after immunosuppressive treatment

Spectratype analysis was performed on PBLs from three HAA patients and compared to the PBL spectratype analysis of healthy volunteers. The ten healthy controls showed predominantly normal or Gaussian spectratype profiles, with only 17% ±12% having a skewed Vβ pattern; the complexity score was 92 (±11). In contrast, the HAA patients showed a highly skewed Vβ pattern (60%±17%) ($P < 0.001$), similar to the Vβ skewing seen in the liver-infiltrating lymphocytes. However, for many of the spectratypes no signal was observed, and the complexity score was markedly reduced 37±13 ($P < 0.001$) (Figure 4).

All three patients were treated by immunosuppressive therapy, with good hematological response. Follow-up samples were available on two patients one year after treatment, and in the third at 2.5 years after treatment. After immunosuppressive therapy many skewed TCR Vβs reversed to a "normal" Gaussian distribution (Figure 5), and many of the Vβs that were previously not detected now gave a spectratype signal. The number of both skewed Vβs and absent Vβs before and after treatment both decreased. Similarly, there was a significant increase in mean complexity scores ($P < 0.01$), with a mean score after treatment of 94 (±8), not different from the complexity scores of the healthy donors (mean score 92±11; Figure 4).

Discussion

Although the clinical characteristics and response to immunotherapy indicate that HAA is immune-mediated,[8] there have been no studies of the T cell repertoire in this

syndrome. However a number of reports have studied the Vβ T cell repertoire in hepatitis B, hepatitis C infection, and autoimmune hepatitis. Bach-Nga *et al*[16] studied the Vβ composition of liver-infiltrating lymphocytes and showed that, in both hepatitis B and C infections, there was a preponderance of certain Vβ families; the Vβ families over represented differed between the two viral etiologies. Spectratype data have confirmed these observations. Sing *et al.* [25] compared the clonality of Vβ T cell receptor–bearing population between liver and peripheral blood in patients with hepatitis B and found clonotypic expansions in 4-9 TCR Vβ subfamilies, indicating a high restriction in the T cell composition of liver-infiltrating lymphocytes. Similar experiments in hepatitis C,[17] and autoimmune hepatitis[18,26] showed many skewed spectratype patterns in lymphocytes in the liver.

Our results from the four cases of chronic hepatitis B and C for TCR spectratyping analysis found a similarly highly skewed Vβ pattern consistent with these published results (and validating our use of frozen tissue). In addition we demonstrated that we could detect a similar skewed spectratype pattern in the livers of patients with HAA. This was in contrast to livers from patients with biliary atresia who, as in healthy livers, should not have an inflammatory or T cell infiltrate. When we also compared the complexity of the T cell repertoire between HAA and viral-hepatitis similar complexity scores were obtained in both diseases. Although we cannot conclude whether the same or different antigens are involved in this broad stimulation, our data are suggestive of a similar pathogenic mechanism, perhaps triggered by an antigen-specific immune response as in viral hepatitis, and supporting the hypothesis based on epidemiological and clinical investigations that an unknown pathogen may be involve in this disease.[27]

Some studies using peptides as antigens have suggested a limited T cell response, with different clones having the same or related CDR3 sequences.[28,29] Shared CDR3 sequences have been harder to identify in hepatitis infections, although putative shared motifs have been suggested.[17,25,26] When we analyzed the specific CDR3 lengths in the TCR Vβ repertoire in these patients, we did not find any common peaks shared by three or more samples. However, our samples were not HLA-matched. The current negative finding nevertheless does not support the role of a superantigen or shared CDR3 sequences in the pathogenesis of HAA.

One of the major limitations of our study was that we were unable to obtain paired liver and peripheral blood samples from the same patients. However, when PBLs from different patients with HAA were examined, we observed a similar broadly skewed T cell repertoire pattern (>50% of Vβ families), as found in the liver samples, with a statistically significant increase in the complexity score of the spectratypes compared to controls, suggesting an antigen-driven TCR repertoire and limited usage of the TCR. In addition, evaluation of the TCR Vβ spectratypes before and after successful immunosuppressive therapy showed reversion to a "normal" spectratyping profile after treatment. This strongly suggests that pathogenic T cells had been eliminated or decreased so as not to be detectable, and a relationship between Vβ changes and an autoimmune pathophysiology.[30]

Our results in the HAA patients can be compared and contrasted with similar data in idiopathic aplastic anemia. Manz *et al*[31] found a restrictive T cell expansion in both bone marrow and PBLs of patients with severe AA, with 1 or 2 oligoclonal Vβ patterns per patient, and suggested that the T cell repertoire expansion was random with respect to

the Vβ chain. Data from a Japanese study[32] showed that patients with cyclosporine-dependent AA had highly skewed Vβ spectratypes, with patients who responded to immunosuppressive therapy still showing skewing, but in less Vβ families (<= 20% abnormal patterns). In an earlier study comparing TCR repertoire at initial presentation and after therapy in AA patients, we found much broader spectratype skewing (44%±33%), with oligoclonal expansions of Vβ15, Vβ21, and Vβ24 in more than 70% of AA patients with HLA-DR2.[33] In contrast to the HAA patients reported here, after immunosuppressive therapy no significant change was found in the degree of Vβ skewing, with patients treated with cyclophosphamide even showing more oligoclonality. Skewed T-cell repertoires[34] were also seen in paroxysmal nocturnal hemoglobulinuria (PNH), a syndrome often associated with AA.[35] In a study of CD4 and CD8 lymphocyte subpopulations in patients with AA and PNH, Risitano *et al*[36] demonstrated that although the abnormal Vβ-distribution pattern was retained after immunosuppressive therapy, the degree of expansion of individual Vβs was lower. For transformed CD4 and CD8 clones obtained from AA patients, Zeng *et al*[37] reported that most CD4 clones displayed Vβ5 and CD8 clones displayed Vβ13, and that ATG and cyclosporine treatment led to marked decrease in clones bearing the dominant CDR3 Vβ5 sequence in HLA-DR2 patients.

Although HAA is usually considered as a subset of AA, HLA restriction patterns may differ (our manuscript in preparation). We have observed no increased association of HLA-DR2 in HAA patients. Differences in T cell repertoire have been observed in other immune mediated diseases, such as multiple sclerosis,[38,39] rheumatoid arthritis,[40,41] and autoimmune hepatitis,[42] where a limited number of T cells using a restricted diversity of the Vβ subfamilies to proliferate dominantly is revealed in different patients,[21] but the

TCR repertoire pattern is different among these diseases due to the different antigenic triggers.[39,43,44]

In summary, we show that in HAA there is an infiltration of both clonal and many non-clonal T cells, giving rise to a markedly skewed T cell repertoire, as seen in both viral and autoimmune hepatitis. This highly skewed T cell repertoire is also evident in the blood at the time of presentation of bone marrow failure. The expanded T cell clones are replaced by a normal Gaussian distribution of T cell repertoire after immunosuppressive treatment, possibly associated with an antigen clearance and/or loss of T cell due to therapy. Further study on the disease-specific T cell clones ($CD8^+$ or $CD4^+$) and their roles in the immunopathology of HAA are undergoing investigation.

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Figure legends

Figure 1

Determination of the sensitivity of the TCR Vβs spectratyping technique. Detection of spectratyping sensitivity was performed based on flow cytometry, and the sorted positive cells were mixed with negative cells to produce fixed proportions of the specific Vβs in negative cell populations (0, 10, 10^2,10^3,$10^4$and 10^5 Vβ positive cells in 10^6 cell population for each Vβ subfamily). Total RNA (~1μg) was extracted from cells (10^6) and standard spectratyping performed. The experiments were repeated 3 times. The lowest cell number detectable in 10^6mixed population is 100 to 1000 cells for random selection of Vβ2, 14 and 22.

Figure 2

Representative TCR Vβ CDR3 size spectratype obtained from the liver of a patient with HAA (patient P) showing many highly skewed spectratypes. Vβ13 and 23 show a normal or Gaussian "bell-shaped" distribution; Vβ7 and 24 demonstrate a skewed spectratype profile with a single dominant peak; Vβ2 and 9 illustrate a skewed spectratype profile with double peaks; Vβ16 shows multiple peaks but with non-Gaussian distribution and skewed spectratype. The complexity score for each spectratype is given in the upper right corner. The overall complexity score was calculated as 70. The abscissa shows CDR3 size length (amino acids) and ordinate shows fluorescence intensity.

Figure 3

Marked T cell skewing in liver infiltrating lymphocytes of HAA patients (n = 7) and hepatitis patients (n = 4). Black box indicates the skewing Vβ profile by observation and RI; gray box indicates the normal Vβ profile; white box indicates no detectable signal. Overall, there was 60% ± 17% skewed spectratypes, with 5% ±12% absent spectratypes in the HAA patients, compared to 67% ± 0% skewed Vβs, with no signal in 11% ± 13% of reactions, in the viral hepatitis patients. Thus, no significant difference was shown in the number of skewed Vβ spectratypes in each group ($p > 0.5$).

Figure 4

Comparison of the T cell repertoire by different methods in ten healthy donors and three HAA patients before and after immunosupression therapy (mean ±1SD), illustrating a return towards a normal T cell repertoire after successful treatment. The percentage of absent and skewed spectratypes in peripheral blood lymphocytes were first evaluated by observation, then the percentage of skewings of Vβ spectratypes were evaluated by the relative fluorescence intensity (RI), and the complexity scores calculated.

Figure 5

Reversion of the T cell repertoire to 'normal' in three HAA patients after immunosuppressive therapy. Samples for case #1 and #2 were collected at 1 year after immunosuppressive treatment and for case #3 at 2.5 years (ATG, cyclosporine (CsA) and mycophenolate mofetil (MMF)). Shown are the CDR3 size length (40 amino acids) in x-axis versus the fluorescence intensity in y-axis.

Tables

Table 1.

Comparison of the Vβ spectratype analysis by different methods in liver infiltrating lymphocytes in HAA and viral hepatitis patients

Patients	N	Absent (observation) %	Skewed (observation) %	Skewed (RI) %	Total Complexity score
HAA	6	4.8±11.7*	59.5±16.7	61.9±17.8	68.3±7.7
Viral hepatitis	3	11.1±12.5	66.7±0	68.3±18.4	69.7±6.5

* mean ± 1SD

The spectratype profiles of the 21 Vβ subfamilies were analyzed in three ways including observation, measuring the relative fluorescence intensity, or complexity scoring. Absent: no signal detected using current technique; skewed spectratype (observation): the percentage of non-'Gaussian' spectratypes as determined by eye; skewed (RI): the percentage of skewed spectratypes using a calculation based on the relative fluorescence intensity of each peak; total complexity score: the total number of discrete peaks in each Vβ subfamily, as a measure of the polyclonality of the T cell repertoire.

Figure 1

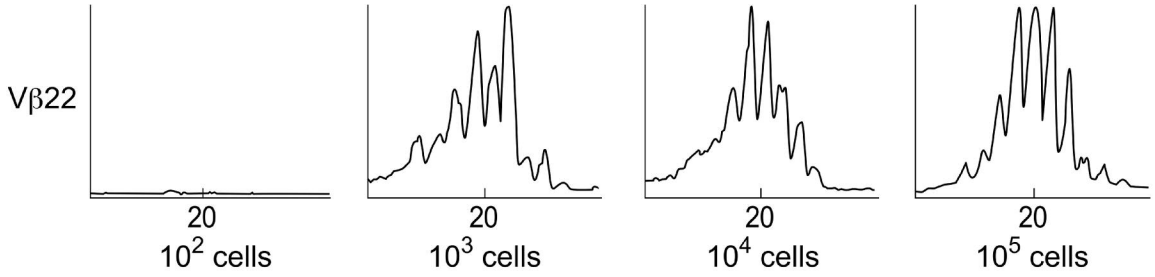


Figure 2

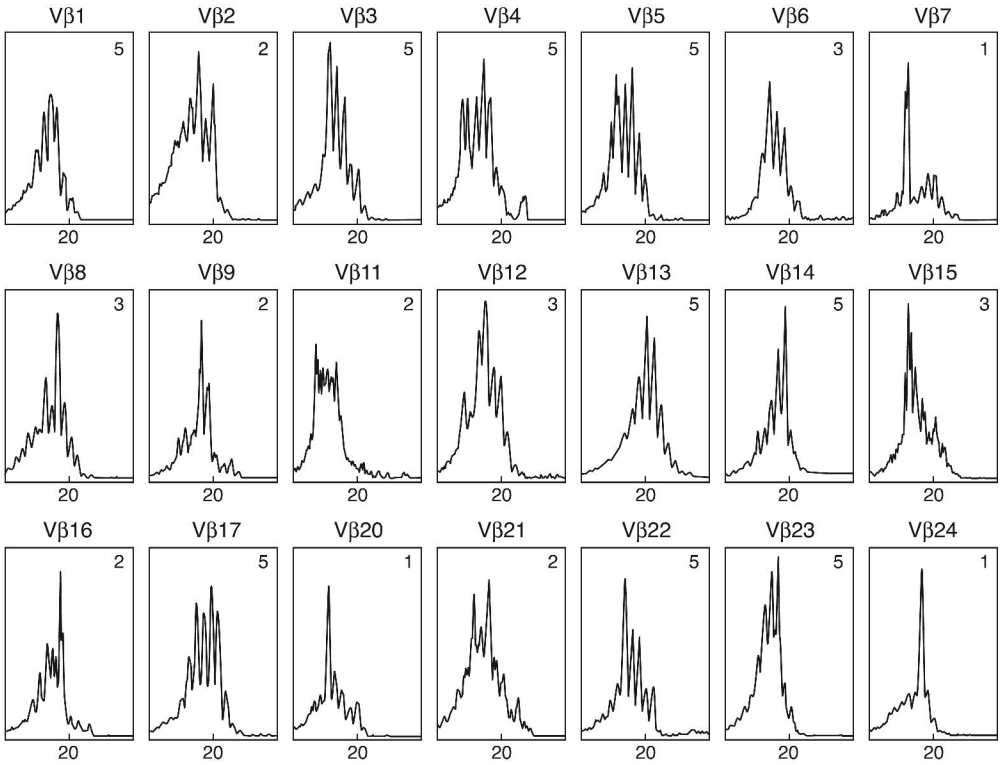

Vβ1
5
20
Vβ2
2
20
Vβ3
5
20
Vβ4
5
20
Vβ5
5
20
Vβ6
3
20
Vβ7
1
20
Vβ8
3
20
Vβ9
2
20
Vβ11
2
20
Vβ12
3
20
Vβ13
5
20
Vβ14
5
20
Vβ15
3
20
Vβ16
2
20
Vβ17
5
20
Vβ20
1
20
Vβ21
2
20
Vβ22
5
20
Vβ23
5
20
Vβ24
1
20

Figure 3

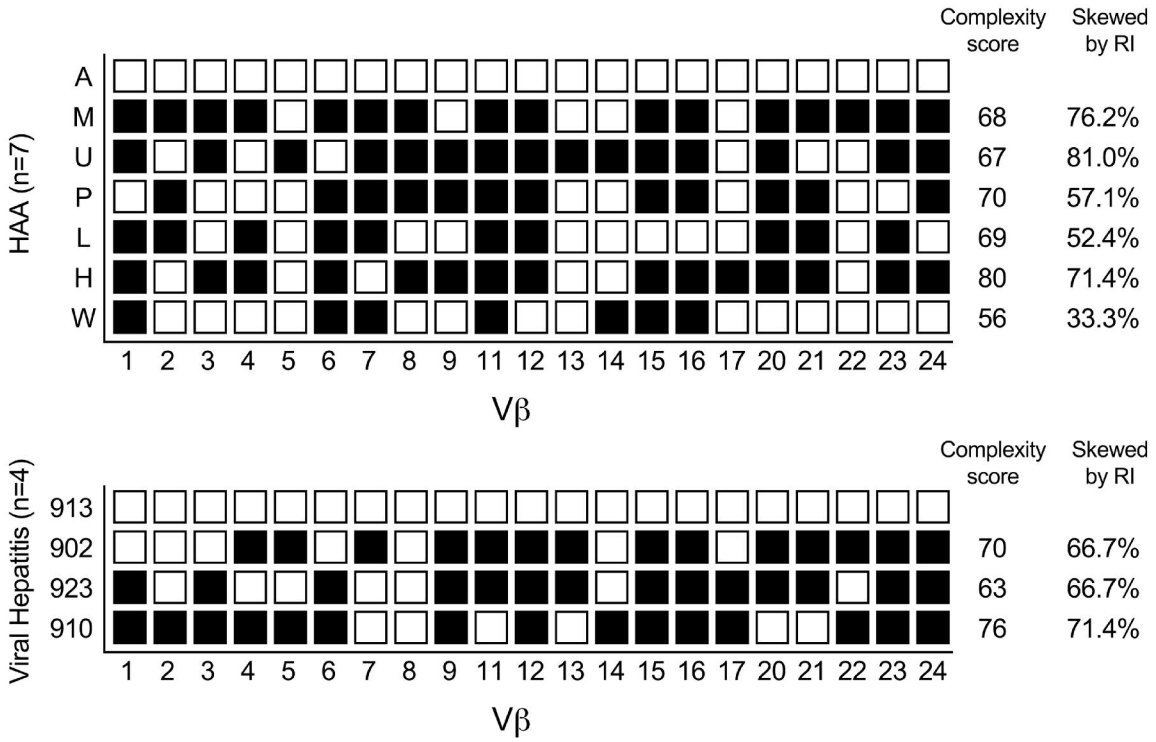

Complexity score
Skewed by RI
HAA (n=7)
A
M
U
P
L
H
W
68 76.2%
67 81.0%
70 57.1%
69 52.4%
80 71.4%
56 33.3%
1 2 3 4 5 6 7 8 9 11 12 13 14 15 16 17 20 21 22 23 24
Vβ
Complexity score
Skewed by RI
Viral Hepatitis (n=4)
913
902
923
910
70 66.7%
63 66.7%
76 71.4%
1 2 3 4 5 6 7 8 9 11 12 13 14 15 16 17 20 21 22 23 24
Vβ

Figure 4

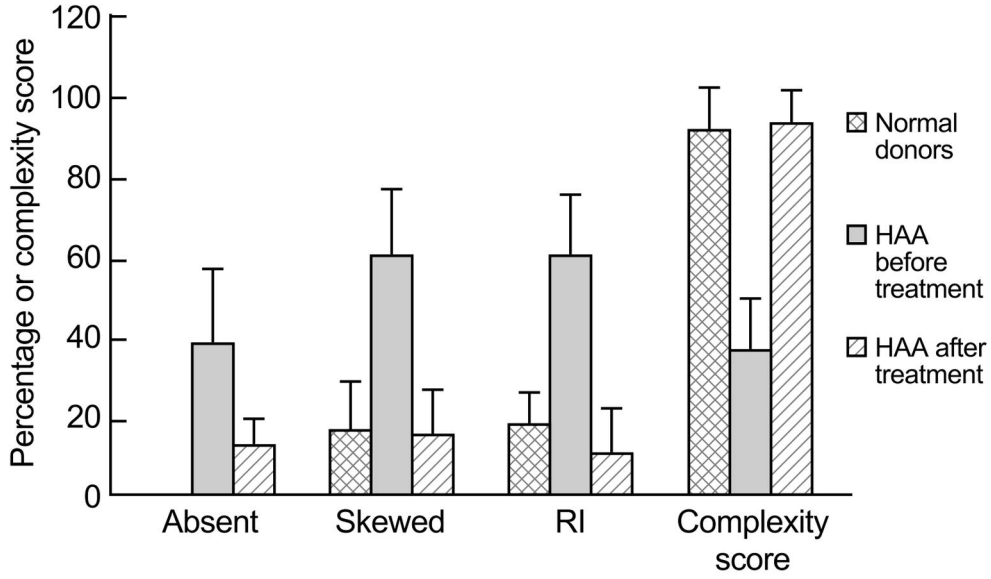
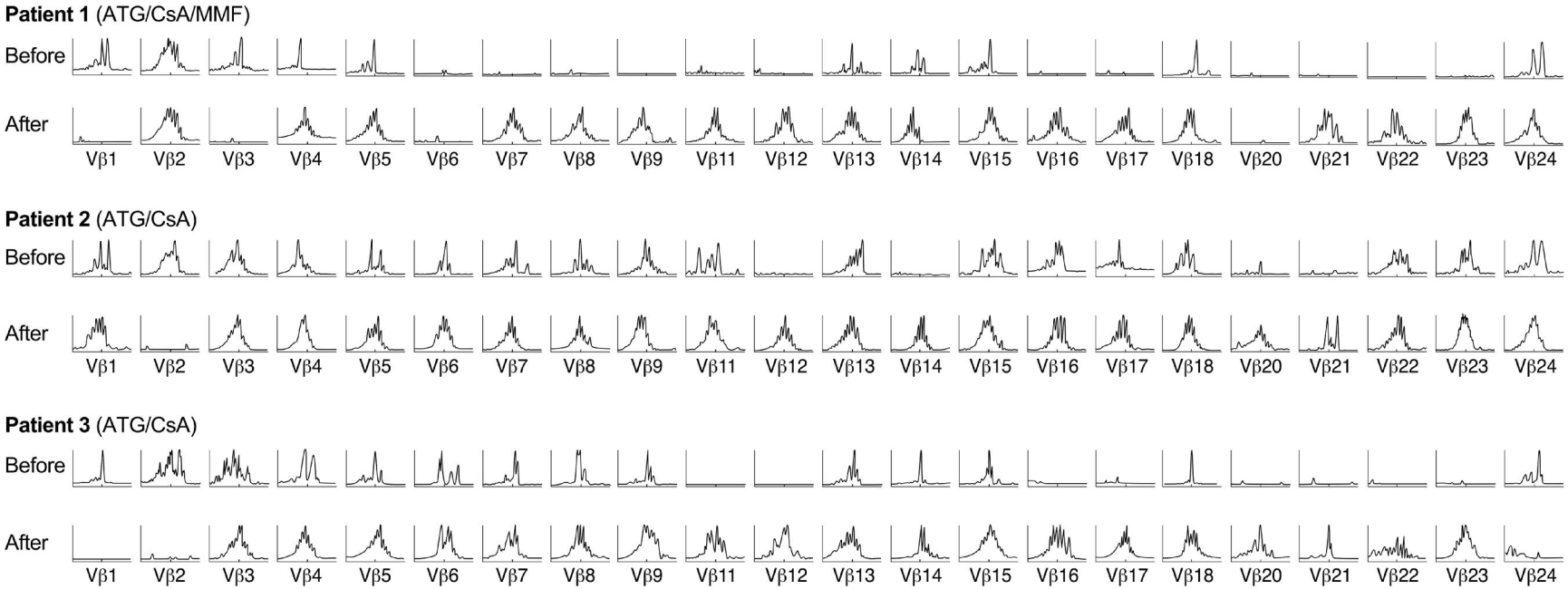

Percentage or complexity score
120
100
80
60
40
20
0
Absent
Skewed
RI
Complexity score
Normal donors
HAA before treatment
HAA after treatment

Figure 5


Patient 1 (ATG/CsA/MMF)
Before
After
Vβ1 Vβ2 Vβ3 Vβ4 Vβ5 Vβ6 Vβ7 Vβ8 Vβ9 Vβ11 Vβ12 Vβ13 Vβ14 Vβ15 Vβ16 Vβ17 Vβ18 Vβ20 Vβ21 Vβ22 Vβ23 Vβ24
Patient 2 (ATG/CsA)
Before
After
Vβ1 Vβ2 Vβ3 Vβ4 Vβ5 Vβ6 Vβ7 Vβ8 Vβ9 Vβ11 Vβ12 Vβ13 Vβ14 Vβ15 Vβ16 Vβ17 Vβ18 Vβ20 Vβ21 Vβ22 Vβ23 Vβ24
Patient 3 (ATG/CsA)
Before
After
Vβ1 Vβ2 Vβ3 Vβ4 Vβ5 Vβ6 Vβ7 Vβ8 Vβ9 Vβ11 Vβ12 Vβ13 Vβ14 Vβ15 Vβ16 Vβ17 Vβ18 Vβ20 Vβ21 Vβ22 Vβ23 Vβ24