Supplementary Discussion

Genes defining 8.1 and 8.2 do not represent extremes of a Gaussian distribution of gene expression.

It was important to determine if groups 8.1 and 8.2 were defined by distinct bimodal gene expression profiles, or if the groups represented the extreme ends of a Gaussian distribution of gene expression. To test this, the AAV cohort was randomly subdivided into two groups, identifying 323 differentially-expressed genes (FDR < 0.05). Expression of these genes across the AAV cohort was compared to a series of idealised template distributions (**Supplementary Fig. 8e**) to determine the closest match. As expected, most genes (75%) were normally distributed across the population, with only 12.4% conforming to a bimodal distribution (**Supplementary Table 7a**). When the same analysis was performed using 1860 genes defining groups 8.1 and 8.2 (FDR<0.05, fold-change>1.5), it was found that 67.3% best fitted a bimodal distribution template, while only 14.3% conformed to a normal distribution (**Supplementary Table 7b**), demonstrating a significant skewing away from the Gaussian distribution in expression of genes differentiating 8.1 and 8.2 ($p < 0.0001$, **Fig. 3e**).

Changes in both naive and memory T cell gene expression are integral to the signature defining the prognostic groups.

We attempted to classify patients into the two subgroups using flow cytometric quantification of T_{MEM} populations. Despite a significant difference in the absolute size and proportion of T_{MEM} populations between subgroups 8.1 and 8.2 these populations overlapped (**Supplementary Fig. 10b, c**) and so could not be used to confidently predict subgroup identity. This implied that gene changes in naive T cells may also contribute. This was supported by three lines of evidence. First, 216 of 1030 genes (21%) characteristic of the T_{EM} population[7] are found in the gene list best defining the difference between 8.1 and 8.2 – broadly similar to the 63 of 369 genes (17%) characteristic of T_N cells [7] **(Supplementary Fig. 10d)**. Second, increased expression of mRNA for the anti-apoptotic molecule Bcl2 was characteristic of group 8.1. Increased expression of Bcl2 protein was similarly seen in the T_{MEM} population but also, more strongly, in the T_N subset (**Supplementary Fig. 10e**). Third, clear bimodal expression patterns were seen for a subset of genes known to be specifically expressed by T_N cells[7] (**Supplementary Fig. 10f-i**). This indicates that these T_N genes are differentially expressed in each of groups 8.1 and 8.2 in the same way as T_{MEM} and other genes, consistent with them being an integral component of the signature rather than being present by chance as the result of being at the extreme of normal distribution of gene expression.

These results taken together provide a model which might explain correlation of the CD8 expression signature with clinical disease, and which is supported, but not proven,

by these data. The variation in gene expression in naive T cells, prior to antigen encounter, seen in group 8.1 may drive increased differentiation into memory T cells, at least in part via IL7R and TCR signalling. This in turn could increase effector T cell generation, tissue damage and thus disease flare (**Supplementary Fig. 11**).

Prognostic subgroups in AAV may be identified using expression of 3 genes

In order to facilitate the translation of these prognostic expression signatures to clinical practice we developed a predictive model using expression data from a smaller number of genes. Robust discrimination of both subgroups in AAV and SLE could be achieved using as few as three target genes (positive predictive value (PPV) 94%, negative predictive value (NPV) 100%, **Fig 4g, h**). The single patient incorrectly classified was the only 'borderline' case, originally classed as 8.1 by one clustering technique and 8.2 by another (Supplementary Fig.1d). Reclassification of this individual as 8.1 would improve rather than weaken the observed association with poor outcome. Multiple gene combinations could be used to classify patients accurately, with the 3 gene classifier illustrated in **Supplementary Fig. 12** chosen by optimising signal-to-noise ratios between the subgroups **(Supplementary Methods)** – it showed no discrimination between the two disease phenotypes (**Fig.4 g**). Interestingly, the three genes included (*ITGA2, PTPN22, NOTCH1*) have all been associated with the development of T cell memory responses and with autoimmunity[9-15]. It should, however, be noted that the accuracy of prediction depended on the use of RNA from purified CD8 T cells and could not be replicated using RNA from un-separated PBMC (**Fig. 4i**).

Subgroups 8.1 and 8.2 may not be discernible after therapy

We sought to determine whether the gene expression signature could predict prognosis in a cross-sectional fashion when applied to patients with inactive disease. 33 AAV patients were rebled 12 months after enrolment, when their disease was in remission on maintenance immunosuppression therapy, and their CD8 T cell transcriptomes compared. The distinctive gene signature that had defined the two subgroups was not preserved (**Supplementary Figure 13a, b)**, though given the intense immunosuppression therapy the patients had received in the interim the significance of this observation remains uncertain. While this lack of consistency does not impinge on the prognostic significance of the gene signature at presentation, of interest when three patients (two v8.1 and one v8.2) were arrayed when re-presenting with a subsequent disease flare, they segregated into the same group as they had at presentation, raising the possibility that the signature might be consistent over time at least in the context of active disease.

Possible factors underlying the gene expression differences in 8.1 and 8.2

That the signature is seen in both AAV and SLE demonstrates that it is not disease-specific. This is underlined by the definition of two subgroups in a population of healthy controls. The full implications of this remain to be determined, but it may underlie some of the variation in propensity to develop a robust CD8 T cell memory response seen within the normal population, something which has long been apparent in vaccination

programmes[16]. It may be, then, that an individual within subgroup 8.1 is not predisposed to the development of autoimmune disease *per se*, but may be more prone to follow a relapsing course once tolerance has been broken and disease develops.

The underlying cause of the gene expression differences between 8.1 and 8.2 is likely to be due to a combination of genetic predisposition and/or antigen exposure. The former is suggested by evidence that genes characteristically expressed in naive CD8 T cells are an important component of the prognostic signature. It is interesting that a number of the genes differentially expressed between the groups are those in which genetic variation is commonly associated with more than one autoimmune disease – genes such as *PTPN22, IL7RA and CD25*[14,17-19]. This supports the concept, emphasised by recent genome-wide association studies, that common critical pathways are involved in multiple autoimmune diseases[20] but it does not necessarily imply that polymorphisms in these genes alone are directly responsible for the 8.1/8.2 division – indeed we have not found associations between the common disease-associated SNPs in *PTPN22*, *IL7RA*, and *CD25* and either group (data not shown). However, while no simple association is apparent it remains possible that variants in these and other genes may contribute in a more subtle polygenic way to the genetic risk for developing an '8.1 phenotype', something that could only be determined by studying a much larger cohort. Alternatively, the expression changes could be driven by an underlying polymorphism in a single unknown transcriptional regulator that favours differentiation into cells with one or other expression signature.

The presence of an ‘8.1’ signature may also in part reflect the context in which a T cell-mediated response has occurred. Vaccination studies have shown that repeated antigen exposure leads to the accumulation of memory CD8 T cells with effector properties[21,22]. The CD8 T_{MEM} expansion seen in subgroup 8.1 could be generated by such enhanced or recurrent priming of memory cells by antigen[23]. Whether this is driven by the nature, frequency or scale of antigen exposure (either directly or via another cell type such as the CD4 T cell) is not clear, but the presence of the subgroups in two autoimmune diseases characterized by responses to distinct autoantigens, as well as in the normal population, suggests it is not driven by a specific autoantigen. Given the potential role played by exposure to antigen in driving CD8 T_{MEM} expansion, it is important to be cautious in extrapolating the results of studies on these subgroups in the normal population to patients presenting with active autoimmune disease.

Supplementary Methods

Patients.

59 AAV patients and 25 SLE patients (plus 1 patient re-enrolled at the time of repeat disease flare) attending or referred to the specialist vasculitis unit at Addenbrooke's hospital, Cambridge, UK between July 2004 and May 2008 were enrolled into the present study. 34 patients presenting with active disease between July 2004 and Oct 2006 composed a prospectively-defined initial vasculitis cohort, while a further 27 patients presenting between November 2006 and May 2008 composed a validation cohort, defined arbitrarily by date of presentation only. Active disease at presentation was defined by Birmingham vasculitis activity score (BVAS[24]) and the clinical impression that induction immunosuppression would be required. Following treatment with an immunosuppressant and tapering dose steroid therapy (**Supplementary Table 2**), patients were followed up monthly for up to 52 months. Of the 59 vasculitis patients who were enrolled into the study 3 were excluded from follow-up analysis due to failure to meet inclusion criteria (x2 concurrent malignancy, x1 non-compliance with maintenance immunosuppression). Repeat analysis with inclusion of these patients did not affect the significance of outcomes presented. Prospective disease monitoring was undertaken with serial BVAS disease scoring[24] and full biochemical, haematological and immunological profiling (**Supplementary Table 1**). At each time-point of follow-up, disease activity was allocated into one of three categories: Flare (at least 1 major or 3 minor BVAS criteria), low-grade activity (0 major and 1-2 minor BVAS criteria) or no activity (0 major or minor BVAS criteria). All disease flares were cross-checked against patient

records to confirm clinical impression of disease activity and the need for intensified therapy as a result. Treatment strategies were recorded (**Supplementary Table 2**). Categorisation of disease activity was performed by a single investigator (EFM) who was blinded to the gene expression-defined subgroup allocation of each patient. Additional flares were defined in the absence of BVAS scoring if patients attended for emergency investigation (bronchoscopy, or specialist ophthalmological or Ear/Nose/Throat surgical review) which confirmed evidence of active disease. To differentiate between discrete flares clear improvement in disease activity was required in the form of an improvement in flare-related symptoms together with a reduction in BVAS score, a reduction in markers of inflammation (CRP, ESR), and a reduction in immunosuppressive therapy.

The SLE cohort was composed of 25 patients attending or referred to the Addenbrooke's Hospital specialist vasculitis unit between July 2004 and May 2008 meeting at least four ACR SLE criteria[25], presenting with active disease (defined below) and in whom immunosuppressive therapy was to be commenced or increased. Following treatment with an immunosuppressant (**Supplementary Table 5**) patients were followed up monthly for up to 1000 days. Disease monitoring was undertaken with serial BILAG disease scoring[26] and full biochemical, haematological and immunological profiling (**Supplementary Table 4**). A timepoint of follow-up was defined as a discrete disease flare if it met all 3 of the following prospectively-defined criteria: 1. new BILAG score A or B in any system, 2. clinical impression of active disease by the reviewing physician and 3. increase in immunosuppressive therapy as a result. Additional flares were defined in the absence of BILAG scoring if patients were admitted directly to hospital as emergency cases for increased immunosuppressive therapy. To differentiate between

disease flares clear improvement in disease activity was required in the form of an improvement in flare-related symptoms together with a reduction in BILAG score and a reduction in immunosuppressive therapy.

Control subjects

40 healthy caucasian individuals, age and sex matched to the disease cohorts were recruited, bled and samples processed in an identical fashion to patient samples. A further 27 healthy individuals were recruited from the National University Hospital in Singapore and again were processed in an identical fashion.

Cell separation and RNA extraction.

Venepuncture was performed at a similar time of day to minimise gene expression differences arising from circadian variation[27]. Peripheral blood mononuclear cells (PBMC), CD4 and CD8 T cells, CD19 B cells, CD14 monocytes and CD16 neutrophils were isolated from whole blood by centrifugation over ficoll and positive selection using magnetic beads as previously described[28]. The purity of separated cell subsets was determined by two-colour flow cytometry. Total RNA was extracted from each cell population using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA quality was assessed using an Agilent BioAnalyser 2100 and quantified using a NanoDrop ND-1000 spectrophotometer.

Naïve CD8 T cell isolation

Naïve CD8 T cells (CD3+8+45RA+62L+) were isolated using a human naïve CD8 T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Microarray hybridisation.

- **Mediante custom spotted microarray**

Total RNA (250 ng) was converted into double-stranded cDNA and labelled with Cy3- or Cy5-dCTP as previously described[28]. Appropriate Cy3- and Cy5-labelled samples were pooled and hybridised to custom spotted oligonucleotide microarrays (mediante) comprised of probes representing 25, 342 genes and control features as previously described[29] All samples were hybridised in duplicate, using a dye-swap strategy, against a common reference RNA derived from pooled PBMC samples. Following hybridisation, arrays were washed and scanned on an Agilent G2565B scanner.

- **Affymetrix Human Gene 1.0 ST microarray**

Aliquots of total RNA (200ng) were labelled using Affymetrix's WT sense Target labelling kit and hybridised to Human Gene 1.0 ST Arrays (Affymetrix) following the manufacturer's instructions. After washing, arrays were scanned using a GS 3000 scanner (Affymetrix).

Data analysis.

Raw image data were extracted using Koadarray v2.4 software (Koada Technology) and probes with a confidence score >0.3 in at least one channel were flagged as present. Extracted data were imported into R where log transformation and background subtraction were performed followed by within-array print-tip Loess normalisation and between-array aquantile and scale normalisation in the Limma package[30], part of the bioconductor project (www.bioconductor.org). Normalised data were then imported into Genespring v7.2 (Agilent) for further analysis. Only data demonstrating a strong negative correlation between dye swap replicates and low level expression of excluded cell specific markers was used in downstream analyses. Affymetrix raw data (.CEL) files were imported into R and subjected to variance stabilisation normalisation using the VSN package in BioConductor.

Differential expression between defined phenotypes was assessed using one-way ANOVA with FDR controlled at 5%. Genes showing minimal variation between defined phenotypes were excluded from analysis using a fold change (FC) filter set at either 1.5 or 2-fold as specified. The degree of overlap between different gene lists was measured in Genespring using the hypergeometric probability function with a specified universe of all T-cell expressed genes. Enrichment of literature-curated gene signatures within different microarray datasets was determined using GSEA[4]. Follow-up analysis of disease activity was performed using the Kaplan-Meier survival method with a log-rank test of significance between groups. Comparisons of outcome and associated clinical variables

between subgroups were analysed using the non-parametric Mann Whitney U test or the Chi-square test as appropriate. The Bonferroni correction for multiple testing was applied to correct for multiple testing where appropriate.

Clustering.

Hierarchical clustering and principal components analysis using an uncentred correlation distance metric and average linkage clustering were performed either in Genespring or Cluster with visualisation in Treeview[31].

Multidimensional scaling and the global test of clustering

Multidimensional scaling was performed in BRB-Array Tools version 3.7.0 Beta_2 release developed by Dr Richard Simon and BRB-Array Tools Development Team[1] and a global test of clustering was applied[32]. Briefly, a series of simulated datasets are generated based on the mean and standard deviation of the top 3 principal components of the experimental dataset, only with univariate Gaussian distributions. The nearest neighbour distance between experimental and simulated specimens as represented in 3-dimensional principal component space is then calculated, along with the empirical distribution function (EDF) of those distances (for any distance, d, the EDF is the proportion of distances that are less-than or equal to d). Data showing clustered 'substrucutre' will demonstrate lower EDF scores, each sample being closer to at least one other sample than with the simulated data. The significance of this may be calculated by comparison with the simulated distribution.

Consensus clustering

Consensus cluster matrices were generated in Genepattern [8] using resampling-based clustering[2]. Briefly, subsamples of a dataset are subjected to repeated rounds of clustering which are summarised in a consensus matrix which indicates the proportion of clustering runs in which two samples cluster together. A distinct consensus matrix is generated for each of the number of clusters to be considered (k=1, 2….n), with the expectation that the most stable subdivision of the dataset will lead to the most robust co-clustering of samples across multiple clustering iterations. Visual inspection and/or interrogation of each consensus matrix thus allows the optimal number of clusters to be defined.

ClusterComparison in ExpressionProfiler:NG

Comparisons between flat and hierarchical clustering methods were performed using ClusterComparison[3] in ExpressionProfiler:NG[33]. Briefly, a hierarchical tree is divided at multiple levels to optimise the correspondence between the resulting clusters and those derived by using a flat (e.g. k-means) clustering algorithm.

Expression density distribution mapping

The CD8 T cell dataset from the AAV patients (n=59) was randomly subdivided in a stratified fashion such that the relative proportions of subgroups 8.1 and 8.2 were preserved in each. Differentially expressed genes (FDR<0.05 and fold-change >1.5) between these randomly-assigned subgroups were identified using ANOVA. This list of differentially expressed genes was then imported into R and the best-fit expression density distribution determined using the BioConductor expression density diagnostics package in R using a neural networks classifier. This procedure was repeated for genes

differentially expressed between subgroups v8.1 and v8.2 in the same cohort of patients. Significant differences in distribution of these genes were determined with a chi-square test comparing bimodal' vs 'other' and 'normal' vs 'other'.

Differential co-expression analysis

The naïve CD8 T cell data for 11 AAV patients from the combined cohort were subjected to differential co-expression analysis using the coXpress package in R[34]. Briefly, hierarchical clustering was performed on the samples (n = 4) from subgroup 8.1 and a threshold of Pearson correlation $r^2 > 0.6$ was applied to generate co-expressed gene subsets. Significance of co-correlation was determined by comparison against co-correlation seen in 1000 genesets drawn randomly from the same dataset. The same process was applied to samples (n = 7) from subgroup 8.2. Only those genesets showing significant ($p<0.05$) co-expression in subgroup 8.1 but not in 8.2 ($p>0.05$) were considered to be co-expressed and were used for further analysis of functional interpretation.

Gene set enrichment analysis and pathway definitions

Genes incorporated in pathways were derived from two existing, independently-curated databases, MSigDB[4] and Ingenuity systems [35]. Alternatively specific signatures were derived from the literature as described. Enrichment of specific pathways was assessed using Gene-Set enrichment analysis (GSEA)[4]. Briefly, all genes from a dataset are ranked in order of their differential expression between two defined phenotypes (in this case 8.1 and 8.2). The distribution of a defined set of genes (such as pathway or signature) within this ranked list is assessed. Multiple random permutations of the actual phenotypes generate a null distribution against which to assess the significance of the enrichment of the gene set in question.

Knowledge-based network generation and pathway analysis

Gene subsets identified by co-expression analysis were further investigated using the Ingenuity Pathways Analysis[35] platform. Briefly, for network analysis genes from a specified target set of interest are progressively linked together based on a measure of their interconnection, which is derived from known functional interactions. Additional highly interconnected genes which are absent from the target genes (open symbols) may be added to complete a network of arbitrary size (set at n = 35). Networks may be ranked by significance which reflects the probability of randomly generating a network of similar size from genes included in the full knowledge database containing at least as many target genes as in the network in question. For pathways analysis, the over-representation of canonical pathways (pre-defined, well-characterised metabolic and signalling pathways curated from extensive literature reviews) amongst a specified set of target genes is assessed, with significance determined by computing a Fisher's exact test with Benjamini-Hochberg correction for multiple testing.

Quantitative RT-PCR.

mRNA levels of *IL7Ra* and *CD69* were determined using Taqman Gene Expression Assays (Applied Biosystems) on an ABI PRISM 7900HT instrument according to the manufacturer's instructions. Transcript abundance was calculated by comparison to a standard curve.

Flow cytometry.

Immunophenotyping was performed using a CyAn ADP flow cytometer (Dako), and data was analysed using FlowJo software (Tree Star). At least 500,000 total events were

collected per sample, reactions were standardised with multicolour calibration particles (BD Biosciences) with saturating concentrations of the following antibodies: PE-Bcl2 (Clone Bcl-2/100, BD Biosciences), APC-CD45RA (Clone HI100, BD Biosciences), PE-Cy5 CD3 (Clone HIT3a, BD Biosciences), PE-Cy7 CCR7 (Clone 3D12, BD Biosciences), PE-CD127 (Clone hIL-7R-M21, BD Biosciences), PE-CD69 (Clone CH/4, Abcam), PE-IL2RA (Clone 143-13, Abcam), Pacific Blue CD8 (Clone RPA-T8, BD Biosciences).

Classifier

CD8 T cell datasets – either complete or using a restricted target gene list as indicated - for each of three populations (AAV, n=59; SLE, n=25; combined n=84) were split into 50% "training" and "test" cohorts in a randomised, stratified fashion. Using a support vector machines algorithm a predictive model was derived from the training set and applied to the test set to assess its predictive performance. Genes incorporated into the optimised classification model were chosen on the basis of favourable signal-to-noise ratio metric between the specified phenotypes, demonstrated expression in CD8 T cells and on their defined roles in CD8 T cell memory. Development of the predictive classifier was performed using the Genepattern 2.0 platform[8].

Ethical approval.

Ethical approval for this study was obtained from the Cambridge Local Research Ethics Committee (Ref 04/023).

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