CD4+CD28+KIR+CD11a^hi T cells correlate with disease activity and are characterized by a pro-inflammatory epigenetic and transcriptional profile in lupus patients

Elizabeth Gensterblum[a,1], **Paul Renauer**[a,1], **Patrick Coit**[a], **Faith M. Strickland**[a], **Nathan C. Kilian**[a], **Shaylynn Miller**[a], **Mikhail Ognenovski**[a], **Jonathan D. Wren**[c,d], **Pei-Suen Tsou**[a], **Emily E. Lewis**[a], **Kathleen Maksimowicz-McKinnon**[b], **W. Joseph McCune**[a], **Bruce C. Richardson**[a], and **Amr H. Sawalha**[a,e,*]

[a]Division of Rheumatology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

[b]Division of Rheumatology, Henry Ford Health System, Detroit, MI, USA

[c]Arthritis and Clinical Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA

[d]Department of Biochemistry and Molecular Biology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

[e]Center for Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI, USA

Abstract

Objective—The goal of this study was to comprehensively characterize CD4+ CD28+ T cells overexpressing CD11a and KIR genes, and examine the relationship between this T cell subset, genetic risk, and disease activity in lupus.

Methods—The size of the CD4+CD28+KIR+CD11a^hi T cell subset was determined by flow cytometry, and total genetic risk for lupus was calculated in 105 female patients using 43 confirmed genetic susceptibility loci. Primary CD4+CD28+KIR+CD11a^hi T cells were isolated from lupus patients or were induced from healthy individuals using 5-azacytidine. Genome-wide DNA methylation was analyzed using an array-based approach, and the transcriptome was assessed by RNA sequencing. Transcripts in the CDR3 region were used to assess the TCR repertoire. Chromatin accessibility was determined using ATAC-seq.

Results—A total of 31,019 differentially methylated sites were identified in induced KIR +CD11a^hi T cells with >99% being hypomethylated. RNA sequencing revealed a clear pro-

*Corresponding author. 5520 MSRB-1, SPC 5680, 1150 W. Medical Center Drive, Ann Arbor, MI 48109, USA. asawalha@umich.edu (A.H. Sawalha).
1These two authors equally contributed to this work.

inflammatory transcriptional profile. TCR repertoire analysis suggests less clonotype diversity in KIR+CD11a^{hi} compared to autologous KIR-CD11a^{low} T cells. Similarly, primary KIR+CD11a^{hi} T cells isolated from lupus patients were hypomethylated and characterized by a pro-inflammatory chromatin structure. We show that the genetic risk for lupus was significantly higher in African-American compared to European-American lupus patients. The demethylated CD4+CD28+KIR+CD11a^{hi} T cell subset size was a better predictor of disease activity in young (age 40) European-American patients independent of genetic risk.

Conclusion—CD4+CD28+KIR+CD11a^{hi} T cells are demethylated and characterized by pro-inflammatory epigenetic and transcriptional profiles in lupus. Eliminating these cells or blocking their pro-inflammatory characteristics might present a novel therapeutic approach for lupus.

Keywords

Lupus; T cells; DNA methylation; Genetic risk; Chromatin accessibility

1. Introduction

Systemic lupus erythematosus is a chronic multisystem autoimmune disease characterized by periods of disease flares and remission. Genetic susceptibility might explain at least in part familial aggregation of the disease, and plays a role in determining age of disease onset, severity, and disease heterogeneity [1–3]. Epigenetic aberrancies also play a role in the pathogenesis of lupus [4]. Lupus T cells are characterized by reduced expression and activity of the maintenance DNA methyltransferase DNMT1, resulting in T cell hypomethylation. Several factors might be mechanistically involved in reduced DNMT1 expression and the methylation defect in lupus T cells [5]. These include defective ERK pathway signaling, and increased expression and activity of PP2A, mTOR, and GADD45a [6,7].

CD4+ T cells from lupus patients are characterized by robust demethylation in interferon-regulated genes [8]. In addition, CD4+ T cells in lupus are characterized by overexpression of several methylation sensitive genes, including CD11a, CD70, Perforin, CD40L, and the KIR gene cluster [9]. Normal CD4+ T cells treated with DNA demethylating agents overexpress these same genes similar to lupus T cells, and become autoreactive *in vitro* [10]. Further, demethylated T cells or T cells with induced defect in DNMT1 expression can cause autoimmunity in animal models [11–13].

Using multi-color flow cytometry, a novel CD4+ CD28+ T cell subset characterized by cell surface CD11a^{hi} and KIR expression was recently identified in patients with active lupus [14]. This T cell subset also expresses other methylation sensitive genes known to be overexpressed on lupus T cells, including CD70 and CD40L. Indeed, treating T cells from normal healthy individuals with DNA demethylating agents results in expansion of this T cell subset [14]. The goal of this study was to characterize this novel T cell subset to reveal the complete repertoire of genes that identify this subset and therefore understand its functional role upon disease pathogenesis. In addition, we aimed to determine if expansion of this T cell subset interacts with genetic risk to predict disease activity in lupus patients.

2. Methods

2.1. Lupus patients

Female participants previously diagnosed with lupus were included in this study. All patients fulfilled the American College of Rheumatology classification criteria for SLE [15]. A Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score was calculated at the clinical visit concurrently with enrollment in the study and blood sampling draw. Patients who had received cyclophosphamide within the past 6 months or who were on methotrexate were excluded from this study, as cyclophosphamide and methotrexate alter the expression of cell surface molecules and DNA methylation patterns, respectively [16,17]. Patients were recruited from the University of Michigan Health System and Henry Ford Health System. The institutional review boards at the participating institutions approved this study. All participants signed an informed consent prior to enrollment.

2.2. CD4+CD28+KIR+CD11a^{hi} T cell subset size measurement

Whole blood samples were separated by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient centrifugation to isolate granulocytes for genotyping and peripheral blood mononuclear cells (PBMCs) for the analysis of the CD4+ CD28+ KIR +CD11a^{hi} T cell subset size. The isolated PBMCs were stained with fluorochrome-conjugated antibodies, fixed with Fixation Buffer (BioLegend, San Diego, CA, USA) and subsequently analyzed by flow cytometry using an iCyt Synergy SY3200 Cell Sorter (Sony Biotechnology Inc., San Jose, Ca, USA) and WinList 8.0 software (Verity Software House, Topsham, ME, USA). The T cell subset size was defined as the percentage of CD3+ CD4+ CD28+ cells expressing both KIR+ and CD11a^{hi} markers. The gating strategy for measuring this T cell subset size is shown in Supplementary Fig. 1.

Flow cytometry staining was performed using the following fluorochrome-conjugated antibodies: APC anti-human CD11a (clone: HI111), APC/Cy7 anti-human CD4 (clone: RPA-T4), Pacific Blue anti-human CD3 (clone: UCHT1) and PE/Cy5 anti-human CD28 (clone: CD28.2) (BioLegend, San Diego, Ca, USA); PE anti-human CD158a,h (clone: EB6B), PE anti-human CD158b1/b2,j (clone: GL183) and PE anti-human CD158i (clone: FES172) (Beckman Coulter, Marseille, France); PE anti-human CD158b (clone: CH-L) and PE anti-human NKB1 (clone: DX9) Becton Dickinson, Franklin Lakes, New Jersey, USA); PE anti-human CD158d (clone: 181703) (R&D Systems Inc, Minneapolis, MN, USA).

2.3. Genotyping and calculation of total genetic risk score for lupus

Granulocytes were isolated from whole blood as described above, and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Genotyping was performed on the Infinium ImmunoArray-24 v2.0 BeadChip (Illumina, San Diego, CA, USA) to assess genetic variation at 39 confirmed lupus risk loci, including 34 risk loci covered by the array and 5 loci in high linkage disequilibrium (LD; $r^2 > 0.9$) with surrogate variants included on the array. An additional 4 lupus risk loci were assessed by TaqMan genotyping assays (Life Technologies Corporation, Carlsbad, CA, USA) using the following probes: rs3768792 (*IKZF2*): reverse 5′-ACCAAAGTCCTACC-CAGAAAACTCT[G/ A]AACTTTCCTGTCTTCCATCATAATT, rs2289583 (*SCAMP5*) forward 5′-

TACAGAGGCATTCATGGGGAGGGAG[A/C] ACTGTTTTTTTTTTACAGATGGGTC, rs887369 (*CXorf21*) forward 5′-CCTTCTGCTCCAGGTACTCATTCAG[A/C]ACTGCATTAGAAATAGGATTCT GCA, rs1734787 (*MECP2*) forward 5′-TTCTCATGGGTACTTTAAGCAG-TAC[A/C]TCTGTTGGCAAAACACAAATGTTGT. Total genetic risk score for lupus was then calculated in each patient as previously described [18] using the formula: $Genetic\ risk = \sum_{i=1}^{43}(n_i \times OR_i)$, where n is the number of lupus risk alleles (0, 1 or 2) and OR is the allelic odds ratio for each lupus risk locus. The genetic variants used to calculate the total genetic risk score are shown in Supplementary Table 1.

2.4. In vitro generation of CD4+CD28+KIR+CD11a^{hi} T cells

We recruited 8 healthy women for these studies. PBMCs were isolated using density gradient centrifugation, and then stimulated overnight with PHA. Cells were then cultured with and without 1 μM 5-azacytidine for 72 h. Magnetic bead separation was used to enrich for CD4+ T cells, then multi-color flow cytometry was used to isolate CD4+CD28+KIR +CD11a^{hi} and CD4+CD28+KIR-CD11a^{low} cells.

2.5. Genome-wide DNA methylation and RNA sequencing

DNA and RNA were extracted and subsequently used to assess the DNA methylome and RNA transcriptome, using the Illumina Infinium HumanMethylation450 BeadChip or Infinium MethylationEPIC BeadChip array, and RNA-seq, respectively. RNA sequencing libraries were created using the TruSeq Stranded mRNA library kit (Illumina, San Diego, CA, USA) from purified total RNA and 100bp, single-end mRNA reads were sequenced on HiSeq 2500 sequencers.

Genome-wide DNA methylation analysis was performed using GenomeStudio Methylation module software (Illumina, San Diego, CA, USA). Raw image files were read into the software and determined to meet QC if at least 99% of probes had a detection *P*-value <0.05. Significantly differentially methylated loci were selected using the following criteria: | Beta| > 10%, detection *P*-value < 0.05, |'DiffScore'| > 22, and no SNP within 10 bp of the probe target site. Raw RNA-seq transcript reads were pre-processed using Trimmomatic (v0.32) and mapped to human genome GRCh38.p2 using STAR (v2.4.2a) [19,20]. Differential expression analysis was accomplished using the exact test in edgeR (v3.6.8) [21]. We selected transcripts with an FDR <0.05 and |fold-change| > 1.5 as being significantly differentially expressed.

2.6. Assay for transposase-accessible chromatin using sequencing (ATAC-seq)

ATAC-seq was performed to assess genome-wide chromatin accessibility as previously described [22,23]. CD4+ CD28+ KIR+ C-D11a^{hi} and autologous CD4+ CD28+ KIR-CD11a^{low} T cell subsets were isolated from 7 female lupus patients. 50,000 cells from each subset were used for the transposition reaction, and DNA libraries were prepared. Following quality assessment as previously described [22], sequencing was performed on a HiSeq2500 Illumina sequencer (2 samples per lane), generating 50bp pair-end reads. Reads were pre-processed to remove adapter reads, and then aligned to the reference genome. Chromatin accessibility peaks were identified and then differential peaks between CD4+CD28+KIR

+CD11a^{hi} and CD4+ CD28+ KIR-CD11a^{low} T cell subsets were identified. Chromatin accessibility peaks with differential accessibility of 1.5-fold or more were annotated and subjected to further analysis. Pre-processing steps and analysis of ATAC-seq data were performed as we previously reported [23].

2.7. Bioinformatics analysis

Functional enrichment analyses were performed using FGNet and GeneTerm Linker Bioconductor packages for the R statistical programming language [24,25], and Database for Annotation, Visualization and Integrated Discovery (DAVID) [26,27]. FGNet was used to create functional gene networks using the results of an initial functional enrichment analysis by GeneTerm Linker, which generated gene-term sets based on ontologies for biological processes, molecular functions, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The functional gene networks were considered significant with an adjusted P value <0.05 and a silhouette width >0, representing a greater functional coherence of genes and gene-term sets. Literature mining was conducted using IRIDESCENT [28–30]. Briefly, the software uses names and synonyms derived from public databases (e.g., OMIM, Entrez, ChemID, Gene Ontology) combined with acronym resolution to identify co-mentioned terms and their frequencies within over 27 million MEDLINE abstracts to develop and score a network of related entities. The distribution of delta-beta values of genes was first plotted and then a cutoff based upon the inflection point was chosen. The inflection point is where the rate of change of the slope reaches zero, suggesting a natural division point between the sites that differ most between conditions. The inflection point was approximately at a delta-beta value of 0.18 and there were 235 genes to the left (i.e., with a delta-beta >0.18). IRIDESCENT then identified the literature relationships between these 235 genes and their relatedness to lupus as a disease.

2.8. TCR repertoire analysis

Sequences containing the CDR3 region of the T cell receptor β chain were extracted from unaligned RNA sequencing reads using MiTCR [31]. Sequences from both the CD4+CD28+KIR+CD11a^{hi} and the CD4+CD28+KIR-CD11a^{low} T cell subsets were extracted, and only sequences with a minimum sequencing quality score of 25 were analyzed. CDR3 sequences were then assigned clonotypes by identifying V and J segments. The TCR repertoire was evaluated based on total number of clonotypes expressed for each sample, and relative expression levels of individual clones were compared between both T cell subsets.

2.9. Statistics analysis

Statistical analysis of the correlation between CD4+CD28+KIR+CD11a^{hi} T cell subset size and SLEDAI score was performed using SAS (v9.4) software (SAS Institute Inc., Cary, NC, USA). European-American and African-American patients were analyzed separately using linear regression and evaluated using a two-tailed t-test. The SLEDAI score was selected as the dependent variable and subset size with and without normalization for genetic risk was the variable of interest. CD4+CD28+KIR+CD11a^{hi} T cell subset size was calculated as a percentage value. The normalized value was calculated as (subset size/genetic risk score)× 100.

3. Results

3.1. In vitro generated CD4+CD28 KIR+CD11a^{hi} T cells are demethylated and characterized by a pro-inflammatory transcriptional profile

We previously demonstrated that CD4+CD28+KIR+CD11a^{hi} T cells express proteins of other methylation sensitive genes known to be hypomethylated in lupus T cells, such as CD70 and CD40L, and that treating CD4+ T cells with the DNA methylation inhibitor 5-azacytidine results in the generation of CD4+ CD28+ KIR+CD11a^{hi} T cells [14]. To assess the extent of DNA demethylation in this T cell subset, we evaluated genome-wide DNA methylation changes in experimentally derived CD4+CD28+KIR CD11a^{hi} T cells compared to autologous CD4+CD28+KIR-CD11a^{low} T cells (n = 8)We identified 31,018 differentially methylated sites across the genome between the two T cell subsets, with 30,736 (99.1%) hypomethylated in CD4+CD28+KIR+CD11a^{hi} T cells. This finding confirms our hypothesis that KIR+CD11a^{hi} expression in CD4+ T cells identifies a demethylated T cell subset, and that this demethylated T cell subset can be distinguished and isolated using flow cytometry. We used literature mining analysis to assess relatedness of the demethylated genes in CD4+CD28+KIR+CD11a^{hi} T cells to lupus. Of 235 genes with a delta-beta of >0.18 used in the analysis, 227 were mentioned at least once in the literature, and 22 of those were mentioned with lupus (P = 0.002, Chi-Square test with Yates correction) (Fig. 1). The relative importance of IL-10 and MMP9 stand out in this analysis, both for the magnitude of their hypomethylation in the KIR+C-D11a[hi] T cell subset and their high interconnectedness within this network. Both *MMP9* and *IL10* genes have been previously shown to be hypomethylated in CD4+ T cells from lupus patients [32,33]. Indeed, treating normal CD4+ T cells with 5-azacytidine resulted in IL10 mRNA overexpression [33].

To determine if DNA demethylation in CD4+CD28+KIR+C-D11a^{hi} T cells is associated with increased transcriptional activity we performed RNA-seq in a subset of the same cells used for DNA methylation analysis (n = 6). RNA-seq identified 1897 differentially expressed genes in the demethylated T cell subset compared to the autologous KIR-CD11a^{low} T cells isolated from the same tissue culture wells. Consistent with the demethylated nature of this T cell subset, 1620 (85.4%) differentially expressed genes were overexpressed in KIR+CD11a^{hi} T cells. Importantly, these RNA-seq data showed significant overexpression of multiple KIR genes including KIR2DL1, KIR2DL3, KIR2DL4, KIR2DP1, KIR2DS4, KIR3DL1, KIR3DL2, KIR3DL3, KIR3DX1 (74.5, 82.6, 37.4, 67.9, 64.9, 42.3, 7.0, 28.7, and 17.2-fold, respectively), which serves as a validation for the RNA-seq data. Other methylation sensitive genes known to be demethylated and overexpressed in lupus T cells were also overexpressed in this experimentally derived KIR+CD11a^{hi} T cell subset, including CD70 (3.3-fold, P = 2.29E-07), and perforin (3.6-fold, P = 2.50E-08). A number of pro-inflammatory cytokine genes, adhesion molecules, Fc-gamma receptor genes, Toll-like receptor genes, HLA molecules, and metalloproteinases were also significantly overexpressed. Of particular interest, and similar to what is known in lupus T cells, the experimentally derived demethylated KIR+CD11a^{hi} T cell subset demonstrates reduced IL2 mRNA expression (3.3-fold, P 0.0079).

We then identified genes that were hypomethylated and also overexpressed in the KIR +CD11a^{hi} T cell subset. These dysregulated genes are likely mechanistically linked to the DNA methylation defect in this T cell subset, and would be expected to help us understand the pathogenicity of this T cell subset as it relates to the DNA methylation defect in lupus. There were 718 hypomethylated and overexpressed genes in the KIR+CD11a^{hi} compared to autologous KIR-CD11a^{low} T cell subset (Fig. 2A). Bioinformatics analysis of these 718 genes revealed significant enrichment in pro-inflammatory gene ontologies, pathways, and gene meta-groups. The most significant gene ontologies enriched in this subset of genes point to a positive regulation of the immune response, and the most significant pathway was "graft versus host disease" (Table 1). Gene meta-group analysis of genes that are both hypomethylated and overexpressed in the *in vitro* generated KIR+C-D11a^{hi} compared to autologous KIR-CD11a^{low} T cells are shown in (Fig. 2B).

3.2. CD4+CD28+KIR+CD11a^{hi} T cell clonality

TCR repertoire analysis, using RNA-seq data, suggests that the KIR+CD11a^{hi} T cell subset is polyclonal, but with less diverse clonotypes compared to autologous KIR-CD11a^{low} T cells (mean number of TCR clones 673 ± 72 versus 837 ± 39; $P = 0.001$) (Fig. 3).

3.3. CD4+CD28+KIR+CD11a^{hi} T cells isolated from lupus patients are characterized by a pro-inflammatory chromatin accessibility pattern

Primary CD4+CD28+KIR+CD11a^{hi} and autologous CD4+CD28+KIR-CD11a^{low} T cells were isolated from 8 female lupus patients (age range: 28–61 years; mean age: 40 years). Genome-wide DNA methylation analysis showed that CD4+CD28+KIR+C-D11a^{hi} T cells isolated from lupus patients were demethylated compared to autologous CD4+CD28+KIR-CD11a^{low} T cells, consistent with our findings in experimentally demethylated T cells. Indeed, out of 37,255 differentially methylated sites between the two T cell subsets, 34,540 sites (93%) were hypomethylated in CD4+CD28+KIR+CD11a^{hi} T cells. To confirm that demethylation in CD4+CD28+KIR+CD11a^{hi} T cells translates to increased chromatin accessibility, we assessed genome-wide chromatin accessibility in CD4+CD28+KIR +CD11a^{hi} T cells isolated from lupus patients compared to autologous CD4+CD28+KIR-CD11a^{low} T cells, using ATAC-seq. We identified 1685 genetic loci with differential chromatin accessibility between the two T cell subsets. 1233 (73%) and 452 (27%) loci demonstrated increased and decreased chromatin accessibility, respectively, in the CD4+CD28+KIR+CD11a^{hi} compared to the CD4+CD28+KIR-CD11a^{low} T cell subset. These data are consistent with overall increased chromatin accessibility in the demethylated CD4+CD28+KIR+CD11a^{hi} T cell subset. Indeed, all differentially methylated CG sites in CD4+CD28+KIR+CD11a^{hi} T cells, which are included on the methylation array and are within a peak of increased chromatin accessibility, were hypomethylated in CD4+CD28+KIR+CD11a^{hi} compared to CD4+CD28+KIR-CD11a^{low} T cells (105 CG sites). Gene ontology analysis of loci associated with increased chromatin accessibility in CD4+CD28+ KIR+CD11a^{hi} cells revealed an overall pattern of a pro-inflammatory phenotype of this T cell subset, further supporting a pathogenic role for this demethylated T cell subset in lupus (Table 2). Of particular interest, chromatin accessibility analysis revealed positive regulation of integrin-mediated cell-cell adhesion in this T cell subset, as well as

positive regulation of cytokine production including IFN-γ, IL-17, and IL-21, but no evidence at the chromatin level to support that these cells can produce Th2 cytokines.

3.4. CD4+CD28+KIR+CD11a^{hi} T cell subset size, genetic risk, and disease activity in lupus patients

DNA methylation defects in T cells play an important role in the pathogenesis of lupus, and T cell DNA demethylation levels correlate with disease activity in lupus patients. In addition, multiple genetic risk loci for lupus have been identified. Therefore, we hypothesized that expansion of the demethylated CD4+CD28+KIR+CD11a^{hi} T cell subset interacts with total genetic risk for lupus to determine disease activity in lupus patients. We determined the size of the demethylated CD4+CD28+KIR+CD11a^{hi} T cell subset by flow cytometry in 105 lupus patients including 68 European-American patients and 37 African-American patients (Supplementary Table 2).

Genotyping across 43 confirmed lupus susceptibility loci was performed to calculate total genetic risk. Genetic risk score was significantly higher in African-American compared to European-American lupus patients in our cohort (7.81 ± 0.12 versus 7.31 ± 0.12 (mean $\pm$ SEM), $P = 0.0079$) (Fig. 4A). As expected, African-American patients had a higher mean SLEDAI score compared to European-American patients (4.19 ± 0.86 versus 1.94 ± 0.29 (mean $\pm$ SEM), $P = 0.003$).

The CD4+CD28+KIR+CD11a^{hi} T cell subset size correlated with disease activity in European-American ($r = 0.25$, $P = 0.038$), but not in African-American lupus patients ($r = -0.18$, $P = 0.29$). Linear regression models suggest that this correlation is largely independent of genetic risk, as the subset size was only a slightly better predictor of disease activity when normalized for total genetic risk in each individual (European-American: $r = 0.27$, $P = 0.024$; African-American: $r = -0.15$, $P = 0.37$). To determine if age influenced the relationship between SLEDAI score and subset size, we assessed age-dependent correlation between the subset size and SLEDAI scores, and show that the CD4+CD28+KIR+CD11a^{hi} T cell subset size, whether normalized to genetic risk score or not, is a better predictor for disease activity in European-American lupus patients 40 years of age (Fig. 4B). Indeed, we find no correlation between disease activity and the subset size in patients >40 years of age (Fig. 4C, Supplementary Fig. 2). No correlation between the subset size and disease activity was detected in any age group in African-American patients (Fig. 4C and Supplementary Fig. 2).

4. Discussion

The role of CD4+ T cell DNA hypomethylation in the pathogenesis of lupus has been thoroughly investigated [4]. We described a demethylated T cell subset characterized by KIR and CD11a overexpression in lupus patients. The KIR locus is comprised of 15 KIR genes, which are most commonly expressed on natural killer cells. However, because regulation of the KIR locus is methylation sensitive, KIR genes have previously been shown to be expressed in the context of lupus [14,34]. Furthermore, KIR expression on CD4+ T cells has been shown to play an active role in T cell cytotoxicity, demonstrated by increased macrophage killing and interferon gamma production by KIR+ T cells, which are both

hallmarks of lupus pathogenesis [34]. In addition, expression of KIR on CD8+ T cells, but not CD4+ T cells has been shown to be correlated with age in healthy individuals. This increased expression of KIR genes on T cells, coupled with the decreased global DNA methylation, could be relevant to the increasing risk of developing lupus with age.

We show that the subset size of CD4+CD28+KIR+CD11a^{hi} T cells is associated with disease activity in young (age 40) European-American, but not African-American women with lupus. We found no correlation between disease activity and the subset size in patients >40 years of age in either ethnic group. This could be due to the known effect of estrogens on inducing oxidative stress [35], which has been linked to DNA demethylation in CD4+ T cells [36]. Indeed, CD4+ T cells exposed to oxidative stress can induce a lupus-like disease in animal models [37]. It is not clear why we did not observe an association between the size of the CD4+CD28+KIR+CD11a^{hi} T cell subset and disease activity in African-American patients. Of interest, African-American lupus patients had a significantly higher genetic risk for lupus compared to European-American patients (Fig. 4A), which is consistent with a higher prevalence and generally more severe disease in African-Americans. This is also consistent with data derived from population based genetic analysis in African and European populations [38]. We should note that the variants used in genetic risk calculation were detected or confirmed in a large European-derived cohort of 7219 lupus patients and 15,991 healthy controls [39]. As of the time of conducting this study, no GWAS data had been published in African-American lupus patients. Ethnicity-specific epigenetic variation could also play a role in the differences between African-American and European-American lupus patients. It has previously been documented that there are significant differences in DNA methylation between African-American and European-American lupus patients in naïve CD4+ T cells [40]. As the CD4+CD28+KIR+CD11a^{hi} T cell subset is characterized by hypomethylation of pro-inflammatory genes, and DNA methylation has been shown to play a role in regulating the KIR locus, this variation could lead to differential KIR expression patterns between ethnicities.

We comprehensively characterized the CD4+CD28+KIR+C-D11a^{hi} T cell subset. When this cell subset is induced *in vitro* via 5-azacytidine treatment, we observed global hypomethylation compared to autologous CD4+CD28+KIR-CD11a^{low} T cells from the same individuals. Moreover, demethylation in CD4+CD28+KIR+CD11a^{hi} T cells coupled with increased chromatin accessibility leads to increase expression of pro-inflammatory genes that could play a mechanistic role in lupus flares.

5. Conclusion

Our findings indicate that the novel CD4+CD28+KIR+CD11a^{hi} T cell subset is demethylated and characterized by pro-inflammatory chromatin accessibility and transcriptional profiles in lupus patients. We identified a number of pathways and specific genes overexpressed in this T cell subset. These data suggest that eliminating CD4+CD28+KIR+CD11a^{hi} T cells or blocking some of the specific pro-inflammatory characteristics we identified might provide novel therapeutic options for lupus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut. 2017.09.011.

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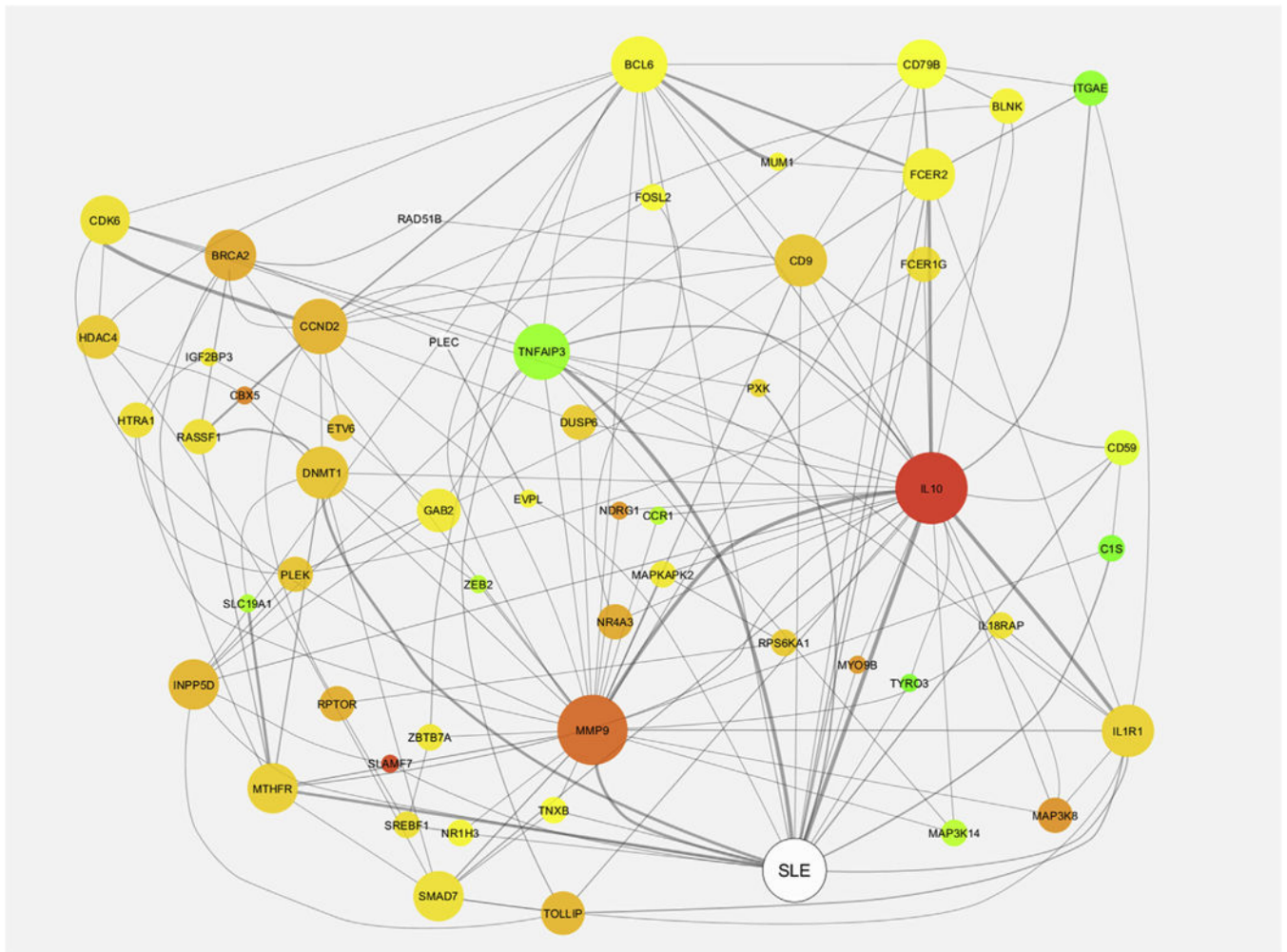
Fig. 1.
Literature relatedness of lupus and the 227 genes with high delta-beta values. The node color corresponds to each gene's relative delta-beta value (higher = red, lower = green). The node size corresponds to how interconnected each gene is within this subnetwork (the larger, the more interconnected), and the edge width corresponds to the strength of the relationship between the entities (the thicker the line, the more literature co-mentions found).
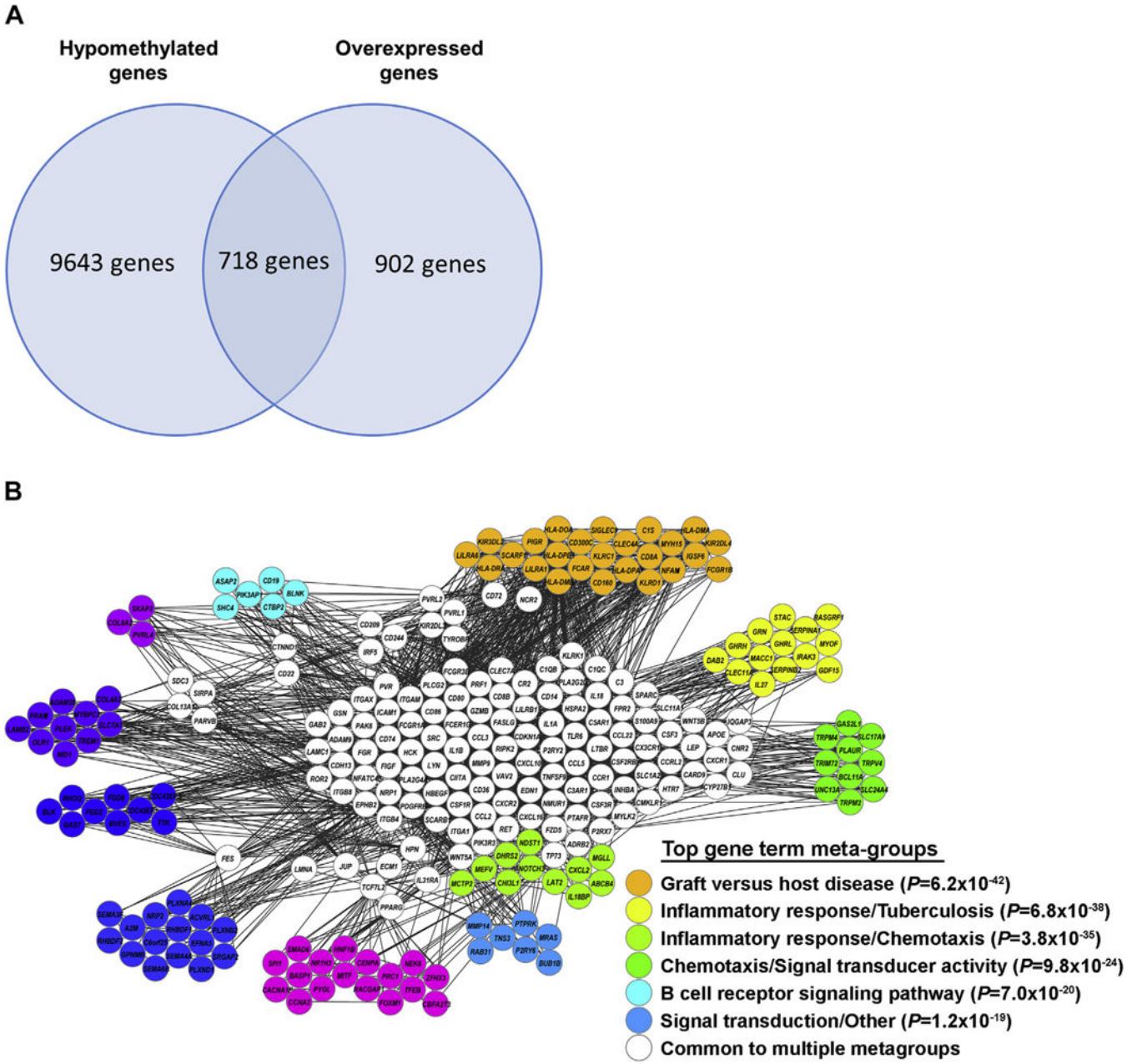
Fig. 2.
(**A**) Number of shared and unique hypomethylated and overexpressed genes in CD4+CD28+KIR+CD11a^{hi} T cells. (**B**) Gene meta-group analysis of genes that are both hypomethylated and overexpressed in the *in vitro* generated KIR+CD11a^{hi} compared to autologous KIR-CD11a^{low} T cells.
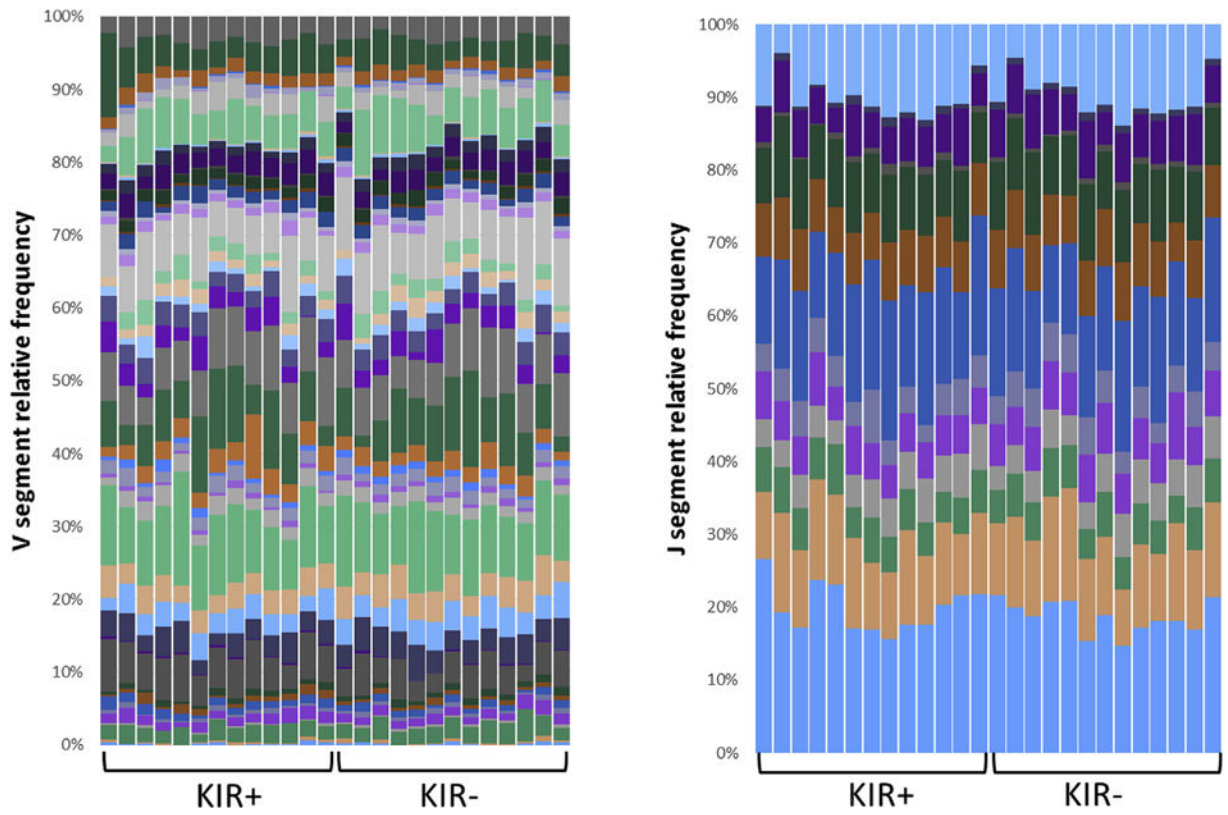
Fig. 3.
TCR V segment and J segment clonotype diversity in *in vitro* generated KIR+CD11a^{hi} compared to autologous KIR-CD11a^{low} T cells. RNA-seq data were used to infer and identify T cell clonotypes from CDR3 sequences. Columns represent individual samples, and the colors in the bars within each column indicate different V and J segments.
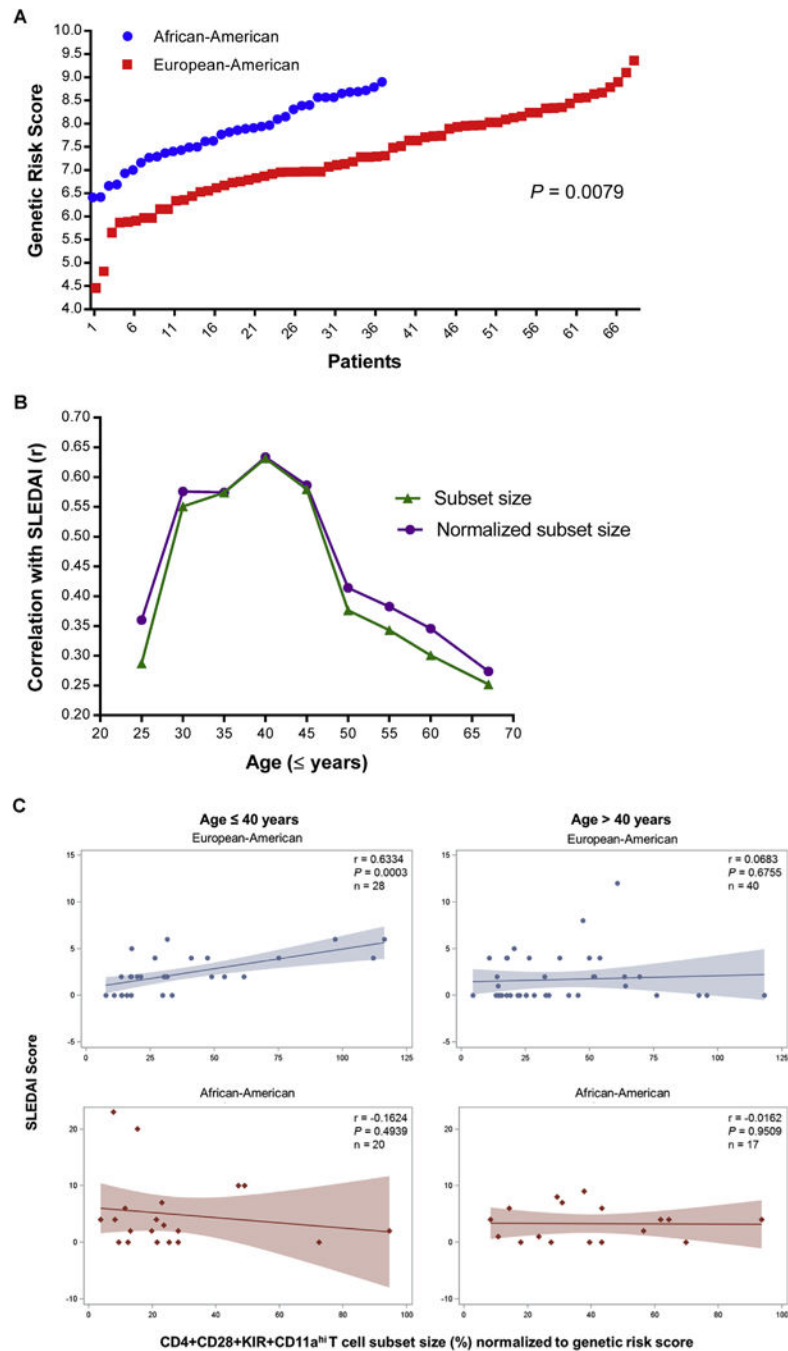
Fig. 4.
(**A**) Genetic risk score for lupus was significantly higher in African-American compared to European-American lupus patients. (**B**) Correlation of SLEDAI score with CD4+CD28+KIR +CD11a^{hi} T cell subset size and CD4+CD28+KIR+CD11a^{hi} T cell subset size normalized by genetic risk score in European-American lupus patients stratified by age (n = 68). (**C**) Correlation of SLEDAI score with CD4+CD28+KIR+CD11a^{hi} T cell subset size normalized by genetic risk score in European-American and African-American lupus patients (age   40

or >40 years) using linear regression. r: correlation coefficient; *P*: two-tailed *t*-test *P*-value. n = number of patients included in regression analysis.

Table 1

Functional annotation analysis of genes hypomethylated and overexpressed in *in vitro* induced CD4+CD28+KIR+CD11a^{hi} cells.

Term	Fold Enrichment	P value (FDR adjusted)
Gene Ontology Analysis		
GO:0002376~immune system process	2.91	4.07E-24
GO:0006955~immune response	3.27	7.53E-22
GO:0006952~defense response	3.17	1.17E-17
GO:0009611~response to wounding	3.36	1.59E-17
GO:0009605~response to external stimulus	2.60	3.67E-16
GO:0006954~inflammatory response	3.95	4.26E-15
GO:0006950~response to stress	1.92	1.28E-11
GO:0048583~regulation of response to stimulus	3.02	2.83E-11
GO:0050776~regulation of immune response	4.00	3.16E-10
GO:0050778~positive regulation of immune response	4.92	5.19E-10
Pathway Analysis		
hsa05332:Graft-versus-host disease	8.59	2.24E-09
hsa04640:Hematopoietic cell lineage	4.35	1.30E-05
hsa04940:Type I diabetes mellitus	6.10	3.02E-05
hsa04612:Antigen processing and presentation	4.04	1.03E-04
hsa05330:Allograft rejection	6.02	2.14E-04
hsa04650:Natural killer cell mediated cytotoxicity	2.96	7.85E-04
hsa05320:Autoimmune thyroid disease	4.25	0.0039
hsa04514:Cell adhesion molecules (CAMs)	2.69	0.0056
hsa05322:Systemic lupus erythematosus	2.99	0.0065
hsa04672:Intestinal immune network for IgA production	4.02	0.0095

Table 2

Gene ontology analysis of genes associated with increased chromatin accessibility in CD4+CD28+KIR +CD11a^{hi} compared to the CD4+CD28+KIR-CD11a^{low} T cells isolated from lupus patients (derived from ATAC-seq). Gene ontologies with fold enrichment of at least 3 and false discovery rate of <10% are shown.

Gene Ontology Term	Genes	Fold Enrichment	P Value	FDR
GO:0033634~positive regulation of cell-cell adhesion mediated by integrin	CXCL13, PODXL, CCL5, PIEZO1	14.24	0.0016	2.95
GO:0032740~positive regulation of interleukin-17 production	PRKCQ, NOD2, IL23R, IL15, LY9, IL21, IL2	9.59	3.75E-05	0.07
GO:0042523~positive regulation of tyrosine phosphorylation of Stat5 protein	IL3, CSF2, IL23R, ERBB4, FYN, GHR, IL2	7.33	2.23E-04	0.41
GO:2000484~positive regulation of interleukin-8 secretion	HYAL2, CD58, F2RL1, CD2, F2R	6.85	0.0047	8.32
GO:0048008~platelet-derived growth factor receptor signaling pathway	PTPRJ, NRP1, BCR, CSRNP1, ARID5B, TIPARP, PDGFC, RAPGEF1, IQGAP1	5.52	1.42E-04	0.26
GO:0015026~coreceptor activity	RGMA, ACVR2A, CD86, NRP1, CD80, CXCR4, CXCR6, TGFBR3, ITGB1	5.34	1.85E-04	0.29
GO:0045672~positive regulation of osteoclast differentiation	FOS, IL23R, KLF10, IFNG, CCL5, TMEM64	5.09	0.0053	9.32
GO:0005164~tumor necrosis factor receptor binding	TRAF2, TNFSF10, TNFSF15, TNFSF14, FASLG, TNFSF9, EDA, TRAF3	4.91	9.03E-04	1.43
GO:0030032~lamellipodium assembly	ARHGEF4, CDH13, GOLPH3, NCK1, RAC1, CAPZB, PARVB, FGD4	4.59	0.0014	2.51
GO:0032266~phosphatidylinositol-3-phosphate binding	DAB2IP, PARD3, PLEKHA5, ZFYVE26, NCF4, ZFYVE28, DENND1A, PHLDA3	4.45	0.0017	2.64
GO:0042110~T cell activation	CD86, CD80, NEDD4, FYN, NCK1, CD2, CD276, TNFSF14, PPP3CA, WAS, DPP4	4.26	1.89E-04	0.35
GO:0005251~delayed rectifier potassium channel activity	KCNC2, KCNQ3, KCNA2, KCNA4, KCNA3, KCNA6, KCNV2, KCNG1	3.95	0.0034	5.32
GO:0032467~positive regulation of cytokinesis	KIF14, CUL3, KIF3B, CXCR5, SVIL, KIF20B, PRKCE, CDC25B	3.75	0.0047	8.33
GO:0007223~Wnt signaling pathway, calcium modulating pathway	GNAO1, GNG2, AGO4, PPP3CA, TNRC6B, PLCB1, TCF7L2, CTNNB1	3.65	0.0054	9.61
GO:0010811~positive regulation of cell-substrate adhesion	LIMS1, HSD17B12, RAC1, CCDC80, NID1, EDIL3, PRKCE, ITGB1	3.65	0.0054	9.61
GO:0045785~positive regulation of cell adhesion	PTPRJ, PTGER4, TNFRSF18, ITGA2, CCL5, NRG1, ADAM8, ADGRG1, TNFSF18	3.41	0.0043	7.60
GO:0014068~positive regulation of phosphatidylinositol 3-kinase signaling	ERBB4, NEDD4, FYN, F2RL1, PDGFC, SEMA4D, CCL5, PRR5L, NRG1, TGFB2, F2R	3.01	0.0032	5.72