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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], 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

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ABSTRACT

Objective: The goal of this study was to comprehensively characterize CD4+CD28+ T cells over-expressing 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-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 $\leq$ 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.

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

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

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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′-ACCAAAGTCCTACCCAGAAAACTCT[G/A]AACTTTCCTGTCTTCCATCATAATT, rs2289583 (*SCAMP5*) forward 5′-TACAGAGGCATTCATGGGGAGGGAG[A/C]ACTGTTTTTTTTTACAGATGGGTC, rs887369 (*CXorf21*) forward 5′-CCTTCTGCTCCAGGTACTCATTCAG[A/C]ACTGCATTAGAAATAGGATTCTGCA, rs1734787 (*MECP2*) forward 5′-TTCTCATGGGTACTTTAAGCAGTAC[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: $|\Delta Beta| > 10\%$, 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

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