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The DNA methylation signature of human TCRαβ+CD4−CD8− double negative T cells reveals CG demethylation and a unique epigenetic architecture permissive to a broad stimulatory immune response

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Abstract T cell receptor (TCR) αβ+CD4−CD8− double negative T cells represent a rare T cell subset implicated in the pathogenesis of several autoimmune diseases. We investigated the DNA methylation signature of double negative T cells to gain insight into the epigenetic architecture of peripheral blood primary human double negative T cells compared to autologous CD4+ and CD8+ T cells. We identified 2984 CG sites across the genome with unique loss of DNA methylation in double negative T cells, and showed significant reduction in mRNA expression of DNA methyltransferases *DNMT1*, *DNMT3A*, and *DNMT3B*. DNA methylation was increased in *CD8A*/*CD8B* and *CD4* consistent with epigenetic repression of both the CD8 and CD4 genetic loci in double negative T cells. We show a consistent increase in non-CG methylation in double negative T cells, a finding suggestive of pluripotency. Network analyses indicate a strong relationship between double negative T cells and functions related to cell–cell interaction, cell adhesion, and cell activation pathways. Our data also suggest a robust pro-inflammatory epigenetic signature in double negative T cells, consistent with a transcriptional permissiveness in key inflammatory cytokines including IFNγ, IL-17F, IL-12B, IL-5, IL-18, TNFSF11 (RANKL), and TNFSF13B (BLYS or BAFF). These findings highlight an epigenetic basis for a role of double negative T cells in autoimmunity.

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1. Introduction

TCRαβ+CD4−CD8− double-negative (DN) T cells represent a rare subset of T cells with a controversial role in the immune system. DN T cell proliferation is significantly increased in the peripheral blood and in involved tissues in several autoimmune diseases [1–5]. In addition, DN T cells exhibit a pro-inflammatory phenotype marked by IL-17 secretion and immune cell recruitment, and have been shown to promote immunoglobulin production in inflamed tissues of patients with autoimmune lymphoproliferative syndrome (ALPS), primary Sjögren's syndrome, and systemic lupus erythematosus (SLE) [1–5]. In contrast, DN T cells of healthy individuals have been described as an immunoregulatory T cell subset able to effectively suppress immune functions of CD4+ and CD8+ T cells [6,7].

DNA methylation, an epigenetic modification that determines chromatin structure and transcriptional accessibility, has a crucial role in determining immune cell function. Importantly, DNA methylation regulates cytokine and transcription factor expression which can determine immune responses and, in CD4+ T cells, differentiation into effector T cell subsets [8]. Further, CREMα- mediated epigenetic remodeling and silencing of CD8 has been recently demonstrated to result in DN T cell expansion [9].

In this study, we characterize the DNA methylome of DN T cells and determine their unique epigenetic architecture by comparing genome-wide DNA methylation patterns in human CD4+, CD8+ and DN T cells from the peripheral blood. Genes hypomethylated in DN compared to both CD4+ and CD8+ T cells were then analyzed for patterns in immune function and regulation using bioinformatic approaches. Our findings show that cell-specific demethylation in DN T cells is highly enriched for immune response genes functioning in cell-adhesion and immune cell activation.

2. Materials and methods

2.1. T cell isolation and DNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples of 7 unrelated healthy women (age range: 25–60) (Supplementary Table 1) by Ficoll-gradient centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Subsequently, T cells were negatively-selected using the Human Pan T Cell Isolation II kit (Miltenyi Biotec, Cambridge, MA). T cells were then analyzed and sorted into TCRαβ+CD4+ T cells, TCRαβ+CD8+ T cells, and TCRαβ+ DN T cell subsets by flow cytometry. DNA was isolated from CD4+, CD8+, and DN T cell subsets using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), then bisulfite-converted using the EZ DNA Methylation kit (Zymo Research, Irvine, CA) for DNA methylation studies.

2.2. Flow cytometry

Flow cytometry was performed using the following antibodies: APC anti-human CD4 (OKT4), Brilliant Violet 711 anti-human CD4 (OKT4), APC anti-human CD56 (HCD56), FITC anti-human CD8a (RPA-T8), Pacific Blue anti-human CD3 (HIT3a), and PE anti-human alpha/beta-TCR (IP26) (BioLegend, San Diego, CA). Analysis and sorting were performed using MoFlow Astrios and Summit software v6.2.3 (Beckman Coulter, Miami, FL). First, live samples were plotted by forward scatter (FSC) and side scatter channels (SSC) then gated on T cells (Fig. 1). Cell aggregates were excluded by gating on T cells plotted by SSC-width versus SSC-height and FSC-width versus FSC-height. For cell purity analyses, T cells were plotted in the Pacific Blue and APC channels to show >97% CD3+ T cell purity using the Pacific Blue anti-human CD3 antibody and >98% CD3+CD56− purity from invariant NK T cells using APC anti-human CD56 (Fig. 1A). For cell sorting, T cells were plotted by side scatter and PE channels to gate TCRαβ+ cells (Fig. 1B). These cells were subsequently plotted by APC and FITC channels to sort CD4+, CD8+, and DN T cells.

2.3. Methylation studies

DNA methylation in isolated CD4+, CD8+, and DN T cells was assessed at >485,000 methylation loci across the genome using the Infinium HumanMethylation450K Beadchip array (Illumina, San Diego, CA). This array interrogates CGs at 96% of UCSC CpG Islands and 99% of RefSeq genes with an average of 17 CG per gene across enhancers, promoterregions, 5′ UTRs, 1st exons, gene bodies, and 3′ UTRs. In addition, the array includes non-CG methylation sites recently shown to undergo DNA methylation in human stem cells.

2.4. Statistical analysis

Analysis of genome-wide DNA methylation in CD4+, CD8+, and DN T cells was performed using GenomeStudio methylation module v1.9.0 (Illumina, San Diego, CA) as previously described[10]. Probe signal-intensities were extracted from image intensity data files. Signal-intensities were normalized to non-CG control probes, and background signals were subtracted based on unhybridized negative control probes. Signal-intensities were then converted to beta values representing methylation levels in a range from 0 to 1. Differential DNA methylation was then evaluated in DN compared to CD4+ and CD8+ T cells from the same individuals. In either T cell comparison, differential methylation was analyzed using the GenomeStudio Illumina custom model described previously [10] and the same normalization parameters listed above. Probes were then filtered for detection P value ($P < 0.05$), which represents detection above background, and probes with SNPs within 10 bp of their 3′ ends were excluded. In either T cell comparison, differentially methylated dinucleotides were defined as sites with an average methylation difference (|delta − beta|) >0.20 and a differential methylation score (|DiffScore|) >33 ($P \leq 0.001$) after adjusting for multiple testing using a Benjamini and Hochberg false discovery rate of 5%. Differential methylation score is 10 * sgn(delta − beta) * −log10(P value). CG location enrichment analysis was performed using Pearson's chi-squared tests with Yates' continuity correction.