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A point mutation in the extracellular domain of CD4 completely abolishes CD4 T cell development in C57BL/6 mouse

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ABSTRACT

In this study, we performed ENU mutagenesis and multi-parameter flow cytometric analysis in C57BL/6 mice to uncover novel genes or alleles regulating immune cell development. We identified a novel mutant allele of *Cd4* gene which completely blocked development of a major subset of T cells named CD4 T cell. Our data for the first time showed experimentally in mice the critical role of the first extracellular domain, by obtaining mice with a loss of function mutation from Ile to Asn at the position 99 of CD4 (I99N). Interestingly, such $CD4^{I99N}$ mutant protein can be expressed on the surface of human cells, and the mRNA stability could be also affected by this point mutation, suggesting that absence of CD4 T cells in mice rooted in the deficiency in function and expression of CD4. In addition, we used this novel CD4 T cell deficient model as recipient mice for adoptive transfer experiment, and showed that it could be an optimal model for study of CD4 T cells.

1. Introduction

Genetic researches are fundamental for understanding the molecular basis of host defense against pathogen and tumor. Even though targeted mutations in mammalian models such as mice give rise to insights into the immune homeostasis and response, forward genetic screening is of particular interest to randomly identify novel genes or alleles that are important for development and function of the immune system (Beutler, 2016). In this study, we performed ENU mutagenesis coupled to multi-parameter flow cytometric analysis in mice. Using exome capture and next generation sequencing, we identified a novel mutant allele of *Cd4* gene that was responsible for the complete absence of CD4 T cell development in thymus. During T cell development in thymus multiple highly coordinated signaling pathways are required for proper thymocytes proliferation and elimination of autoreactive T cells, which include those via T cell receptor, costimulatory molecules, and the co-receptors such as CD4 (Malissen and Bongrand, 2015; Rahemtulla et al., 1991). Structural studies showed that multiple extracellular domains of CD4 exist on T cell surface, including the first extracellular domain that interacts with its ligand MHCII (Wang et al., 2001a). However functional studies elucidating how the extracellular domain maintain its interaction with MHCII, on the basis of *in vivo* mouse models, are still limited. Our data for the first time showed experimentally in mice the critical role of first extra-cellular domain, and a loss of function mutation from Ile to Asn at the position 99 of CD4. Interestingly, this novel $CD4^{I99N}$ mutation was not structurally located in the known interface where CD4 and its ligand interact, but presumably destroyed the three-dimensional structure from the center through alteration of the hydrophobic interaction among those critical amino acids. We further sought to determine whether the mutant allele affected gene expression or function of the protein. Our data showed that such $CD4^{I99N}$ mutant protein can be expressed on the surface of human cells, suggesting that absence of CD4 T cells in mice rooted in the deficiency in function and expression of CD4. In further experiments, we used this novel CD4 T cell deficient model as recipient mice for adoptive transfer study, and the results showed that it could be an optimal model for study of CD4 T cells.

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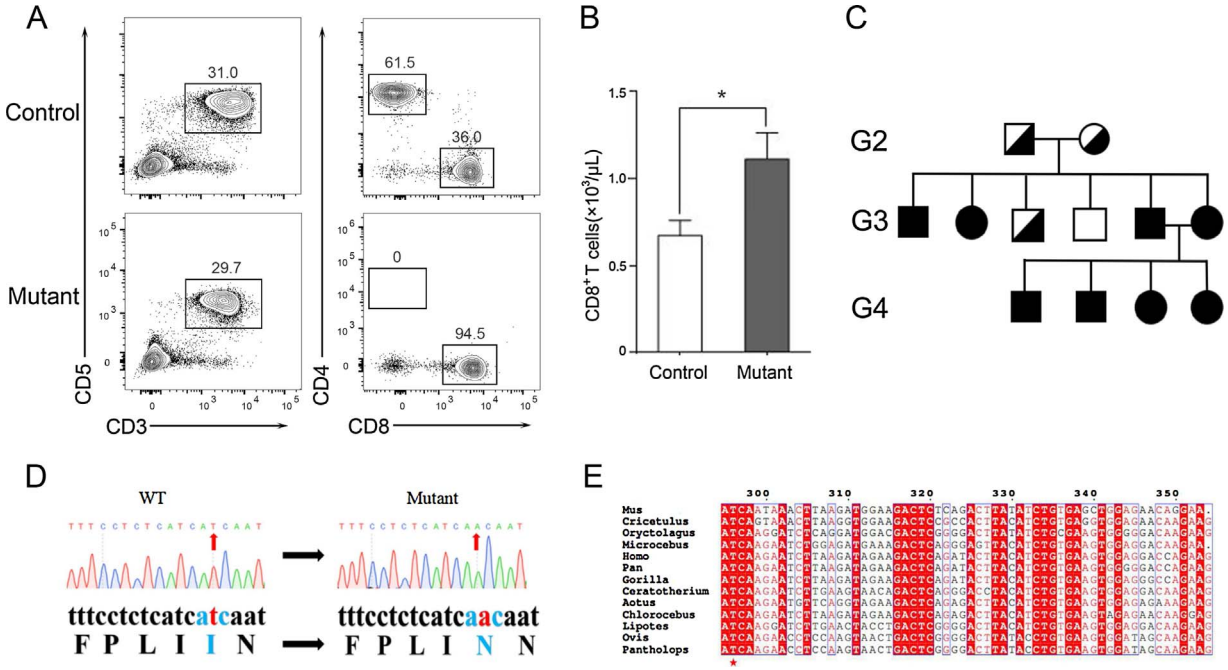

Fig. 1. Identification of a mutant line absent in CD4 T cells in peripheral blood.

A. Mice aged 8 weeks were retro-orbitally bled with anti-clot treated capillaries and the blood samples were labeled with anti-CD3 and CD5 to define T cell compartment. In the T cell compartment, CD4 expressing T cells and CD8 expressing T cells were further analyzed with fluorescent antibodies. No CD4 positive cells were found in the T cell compartment in the mutant mice.

B. The absolute number of $CD8^+$ T cells per microliter of blood were averaged from age-matched wild type control (white) and mutant (grey) mice that were housed in the same condition. Data are shown as mean ± SEM of five mice per group and are representative of three experiments. *$P \leq 0.05$, unpaired Student's *t*-test.

C. Intercross of G2 animals resulted in phenotypic mice absent in $D4^+$ T cells and mice with normal phenotype in G3. Further crossing between the phenotypic mutant mice produced progeny that all express the mutant phenotype of $D4^+$ T cell absence. Determination of wildtype and heterozygotes shown in the figure was obtained in further experiments when the affected gene was identified. Square symbols are male and circle symbols are female. Half-filled symbols indicate heterozygotes, filled symbols represent mutant subjects, and empty symbol indicates wide-type subject.

D. Thymine to adenine transversion resulted in coding alteration from Ile to Asn in mutant mice. The mutation was a homozygous transversion of T to A at nucleotide position 296 in exon 4 of the C4 gene. The mutation located in the first extracellular domain of C4 was substitution of an asparagine for an isoleucine (I99N).

E. Multiple sequence alignment of 13 *Cd4* sequences from Mus musculus and the homologues, including NM_013488.2 (*Mus musculus*), XM_003496094.1 (*Cricetulus griseus*), NM_001082313.2 (*Oryctolagus cuniculus*), XM_020287666.1 (*Microcebus murinus*), NM_000616.4 (*Homo sapiens*), NM_001009043.1 (*Pan troglodytes*), XM_004052582.2 (*Gorilla*), XM_014793995.1 (*Ceratotherium simum*), KR902343.1 (*Aotus nancymaae*), KY225914.1 (*Chlorocebus sabaeus*), XM_007469401.1 (*Lipotes vexillifer*), NM_001129902.1 (*Ovis aries*) and XM_005957883.1 (*Pantholops hodgsonii*). The highly conserved bases are marked with white character in red box, and the T296 of *CD4* is indicated by the red five-point star under the sequence.

2. Materials and methods

2.1. Animals and ENU mutagenesis

Thirty male C57BL/6 mice (G0) aged 8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were housed in the SPF level environment and handled under ethical approval of Xinxiang Medical University. C57BL/6 male mice were injected with three dose of 90 mg/kg ENU (Sigma, N-3385) intraperitoneally once a week for three consecutive weeks. 7 weeks after the last injection, each male mice was crossed to one C57BL/6 female mouse to produce the first generation progeny (G1). Brother-sister mating of G1 mice were performed to produce G2 animals, which were then intercrossed to produce G3 mice for phenotyping. Ly5.1 Foxp3-eGFP knockin mice and CD3e knockout mice were kind gifts from Dr. Marie Malissen at Centre d'Immunophénomique, Marseille, France.

2.2. Immunophenotyping by flow cytometric analysis

Around 100 μL peripheral blood of G3 mice were collected with anticoagulants. 30 μL blood was stained in total volume of 60 μL as described previously (Liang et al., 2013). The panel included fluorescence-labeled antibodies against antigens: CD45 (APC-eFlour780), CD5 (eFlour450), CD3e (Alexa700) CD4 (PE-cyanine7), CD24 (APC-eFlour780) and TCRβ (FITC) from eBioscience; CD8a (BB515) from BD. 400 μL BD FACS™ lysing solution dilluted by distilled water was used for lysing erythrocytes at room temperature for 10 min. Cells were analyzed with flow cytometry BD FACS Canto™. Absolute count of cells was performed by the BD Accuri® C6 with microprocessor-controlled peristaltic pump system (www.bdbiosciences.com/documents/webinar_120512_cellcounting.pdf). The counting was based on gating on the CD45 positive cells that aids in removing debris and enumerating all the white blood cells of the lyphoid organs and blood cells. The same samples were run in parallel using BD FACS Canto™ system by staining more fluorecent antibodies to obtain absolute count for each subset of cells.

2.3. Plasmid construction

To express mouse wildtype CD4 or CD4 with a mutation which replaced isoleucine 99 with asparagine (I99N) in cell line, coding sequence flanked with restriction sites NheI and EcoRI was synthesized (http://www.bioligo.cn). Wildtype CD4 or $CD4^{I99N}$ coding sequence was digested by NheI and EcoRI (NEB) and then cloned into the same multiple cloning sites of vector pCI-neo (Promega, E1841), yielding an intermediate plasmid pCI-CD4 or pCI-mCD4. To facilitate monitoring of CD4 (wildtype or I99N mutant) expression, a fragment containing T2A-EGFP (Thoseaasigna virus 2A peptide) of plasmid PX458 (Addgene #48138) was digested with EcoRI and cloned into the same cloning site of vector pCI-CD4 or pCI-mCD4. Colony-PCR and restriction enzyme