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The artificial loss of Runx1 reduces the expression of quiescence-associated transcription factors in CD4$^+$ T lymphocytes

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ABSTRACT

The Runx1 transcription factor cooperates with or antagonizes other transcription factors and plays essential roles in the differentiation and function of T lymphocytes. Previous works showed that Runx1 is expressed in peripheral CD4$^+$ T cells which level declines after T cell receptor (TCR) activation, and artificial deletion of Runx1 causes autoimmune lung disease in mice. The present study addresses the mechanisms by which Runx1 contributes to the maintenance of peripheral CD4$^+$ T cell quiescence. Microarray and quantitative RT-PCR analyses were employed to compare the transcriptome of *Runx1* −/− CD4$^+$ T cells to those of unstimulated and TCR-stimulated *Runx1* +/− cells. The results identified genes whose expression was modulated similarly by Runx1 deletion and TCR activation. Among them, genes encoding cytokines, chemokines, and Jak/STAT signaling molecules were substantially induced. In Runx1-deleted T cells, simultaneous increases in *Il-17A* and *Ror*γ*c*, a known master gene in T_H17 differentiation, were observed. In addition, we observed that the loss of Runx1 reduced the transcription of genes encoding quiescence-associated transcription factors, including *Foxp1*, *Foxo1*, and *Klf2*. Interestingly, we identified consensus Runx1 binding sites at the promoter regions of *Foxp1*, *Foxo1*, and *Klf2* genes, which can be enriched by chromatin immunoprecipitation assay with an anti-Runx1 antibody. Therefore, we suggest that Runx1 may activate, directly or indirectly, the expression of quiescence-associated molecules and thereby contribute to the maintenance of quiescence in CD4$^+$ T cells.

1. Introduction

In the absence of antigen stimulation, peripheral CD4$^+$ T cells remain naïve and quiescent, and their pool is maintained by homeostatic mechanisms. Upon stimulation by peptide-MHC complex, the activated T cells start proliferating vigorously, secreting cytokines and differentiating into various helper subsets. It has been assumed that the quiescent stage is a default choice for CD4$^+$ T cells and that it is maintained throughout their life as long as the cells do not encounter any matched antigen. However, in recent years, evidence has accumulated showing that quiescence is in fact tightly controlled by various mechanisms (Aiello et al., 2013). One mechanism is intrinsic control by transcription factors. For example, Elf4 (Yamada et al., 2009), Foxo1 (Kerdiles et al., 2009), Foxp1 (Feng et al., 2011), Klf2 (Buckley et al., 2001; Kuo et al., 1997), Slfn2 (Berger et al., 2010), Tsc1 (Yang et al., 2011), and Tob (Tzachanis et al., 2001), as well as ubiquitination-associated molecules such as Peli (Chang et al., 2011) and the COP9 signalosome (Menon et al., 2007), con-

Abbreviations: ChIP, chromatin immunoprecipitation; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genome; RIN, RNA integrity values; TCR, T cell receptor; T_H, helper T cells.

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tribute to the quiescence of cells. Another mechanism is extrinsic control by regulatory T cells (Sakaguchi, 2000). Loss-of-function mutations in the genes or cells mentioned above lead $CD4^+$ T cells to drift out of quiescence into a semi- or hyper-activated stage, eventually resulting in autoimmune diseases.

Runx1 plays fundamental roles in hematopoiesis (Okuda et al., 1996) and is involved in multiple functions throughout T cells lifespan (Wong et al., 2011a). Runx1 is clinically important because gene mutations afflicting *Runx1* gene, such as t(8,21) or t(12,21), are associated with acute myeloid leukemia and childhood lymphoblastic leukemia (Blyth et al., 2005). Runx1 is a ~50 kDa protein encoded by a gene which comprises 7 exons and resides at human chromosome 21q22.12 (Levanon and Groner, 2004). Runx1 belongs to the Runx family of transcription factors (consist of three members i.e., Runx1, Runx2 and Runx3) that share a conserved Runt DNA-binding domain. Through binding of the Runt domain to target genes, the Runx factor activates or suppresses gene transcription depending on the promoter/enhancer context. In addition, at the protein level, Runx1 cooperates with or antagonizes other transcription factors and plays essential roles in T lymphocyte functions (Wong et al., 2011a). For example, Runx1 inhibits helper T cell 2 (T_H2) and induces T_H1 differentiation by repressing Gata3 (Kohu et al., 2009), maintains Treg cells by interacting with Foxp3 (Ono et al., 2007), and induces T_H17 by interacting with Rorγt and FoxP3 (Lazarevic et al., 2011; Zhang et al., 2008). Furthermore, Runx1 is involved in early T cell development in the thymus. Reduction of Runx1 transcription factor activity enhances the apoptotic sensitivity of double-positive thymocytes, whereas Runx1 over-expression impairs the development of thymocytes from the double-negative to double-positive stages (Abe et al., 2005; Wong et al., 2010). In addition, Runx complex is involved in *Cd4* gene silencing in thymus (Jiang et al., 2005; Taniuchi et al., 2002) through interaction with Th-pok (Setoguchi et al., 2008). As a consequence, heterogenous loss of *Runx1* (coupled with *Runx3* deletion) leads to expression of CD4 coreceptor on $CD8^+$ T cells (Woolf et al., 2003). In mature T cells, deletion of *Runx1* gene in *Cd4*-Cre loxP system causes loss of $CD4^+$ T cell population due to diminished interleukin 7 receptor signal (Egawa et al., 2007).

Recently, Runx1 has been implicated in the control of hematopoietic stem cell quiescence (Swiers et al., 2010; Wang et al., 2010). By analogy and based on our previous studies, we speculate that Runx1 may function in the control of naïve T cell quiescence. One reason for this hypothesis is the fact that Runx1 levels are high in naïve T cells but reduced in activated T cells (Wong et al., 2011b). Another reason is the occurrence of autoimmune lung inflammatory disease in mice harboring Runx1-deficient $CD4^+$ T cells (Wong et al., 2012) (we note that the observed phenotype is not due to some impairment of extrinsic Treg cells but due to dysregulation of intrinsic transcription control). These cells exhibit a hyper-activated phenotype ($CD44^+CD62L^-CD69^+$) and secrete elevated levels of cytokines, including Il-2. From these observations, we hypothesized that Runx1-deficient $CD4^+$ T cells are prone to be activated even in the absence of exogenous antigen stimulation.

The purpose of the present study is to address this hypothesis from a genome-wide perspective and to deduce the role of Runx1 in the control of $CD4^+$ T cell quiescence. Since the authentic function of Runx1, as a transcription factor, should be regulation of gene expression, we compared the transcriptome of Runx1-deficient $CD4^+$ T cells with those of naïve and activated Runx1-positive cells. Because the germinal deletion of *Runx1* in mice is embryonically lethal (Okuda et al., 1996), a conditional knockout mice (*Runx1*$flox^{/flox}$) system (Ichikawa et al., 2004) was mated with *Cd4*-promoter driven *Cre* recombinase transgenic mice (Lee et al., 2001) to delete *Runx1* in $CD4^+$ T cells, and the mice were further crossed with *Bcl2* transgenic mice (Strasser et al., 1991) to rescue the total T cell numbers. As expected, the expression of many interleukins and chemokines was up-regulated by Runx1 deficiency. Unexpectedly and most interestingly, Runx1 deficiency caused a reduction in the expression level of genes encoding quiescence-related molecules, such as the transcription factors *Foxo1*, *Foxp1*, and *Klf2*, whose loss induces $CD4^+$ T cell auto-activation. In addition, Runx1 deficiency triggered *Rorγt* induction, a master gene in T_H17 differentiation.

2. Materials and methods

2.1. Mice

Conditional $Runx1^{f/f}$ mice (exon 5 flanked by two loxP sites) (Ichikawa et al., 2004), CD4-Cre-tg mice (Lee et al., 2001) and $Bcl2^{tg}$ mice (B6.Cg-Tg[BCL2]25Wehi/J) (Strasser et al., 1991) were described previously. Mice were crossed to obtain $Runx1^{f/f}$/CD4-Cre-tg/$Bcl2^{tg}$ mice (Runx1 −/−) or control $Runx1^{+/f}$/CD4-Cre-tg/$Bcl2^{tg}$ mice (Runx1+/−). Mice (6–8 months of age) were used for analysis. The control Runx1 +/− mice were apparently healthy, whereas the Runx1 −/− mice showed tachypnea, a hunched posture, suffered from general weakness and lost body weight (Wong et al., 2012). The abnormalities observed in −/− mice are due to the occurrence of autoimmune lung inflammatory disease.

As an inducible gene-targeting system, *Rosa26-CreERT2* mice (B6.129-Gt(ROSA) 26Sortm1(cre/ERT2) Tyj/J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). *Rosa26-CreERT2* and $Runx1^{f/f}$ mice were crossed to obtain $Runx1^{f/f}$; *Rosa26-CreERT2* or control $Runx1^{f/w}$; *Rosa26-CreERT2* mice. To try to delete *Runx1*, mice were injected with 6 mg tamoxifen in a sun flower seed oil (600 μl from 10 mg/ml stock). Mice were sacrificed after eight days. All mice were kept in a pathogen-free environment and handled in accordance with the Regulations for Animal Experiments and Related Activities of Tohoku University.

2.2. Cell isolation and stimulation

The procedures were described previously (Wong et al., 2011a). Briefly, spleens were excised from mice and the cells were suspended in phosphate-buffered saline (PBS) containing 1% (v/v) fetal bovine serum (FBS). Cells were treated with erythrocyte lysis buffer and washed. $CD4^+$ T cells were then isolated by negative selection using the CD4 mouse T lymphocyte enrichment set DM (BD Biosciences, San Diego, CA).

For stimulation, 1×10^6 $CD4^+$ T cells/ml were resuspended in RPMI 1640 medium supplemented with 10% (v/v) FBS, 10 mM Hepes (pH 7.4), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 1× non-essential amino acids, and 50 μM 2-mercaptoethanol. Cells were centrifuged briefly at 700 × *g* for 10 min onto anti-CD3ϵ (5 μg/ml)- and anti-CD28 (2 μg/ml)-coated plates, and incubated at 37 °C in a humidified incubator with 5% CO_2 for 24 h. EL4 cells were cultured in the medium mentioned above.

2.3. Flow cytometry analyses

Spleens were excised and dispersed, and single cell suspension was obtained. Cells were adjusted to 1×10^6/ml and stained with FITC-CD3ϵ (BioLegend, San Diego, CA), PE-CD40L (BD Pharmingen, San Jose, CA), PE-CD44, and allophycocyanin-CD4 (eBiosciences, San Diego, CA). For intracellular staining of cytokines, 1×10^6/ml $CD4^+$ T cells were prepared from splenocytes using anti-mouse CD4 Magnetic Particles-DM (BD Biosciences) and stimulated with 200 ng/ml PMA and 1 mM ionomycin in the presence of 2 mM monensin for 5 h. Cells were processed using Foxp3/Transcription Factor Staining Buffer Set (eBiosciences) and stained with FITC-IFN-γ, PE-IL-4, PE-IL-17A (BD Biosciences), or PE-Foxp3 (eBiosciences) antibodies.