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Regulation of SATB1 during thymocyte development by TCR signaling

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ABSTRACT

T lymphocyte development and differentiation is a multi-step process that begins in the thymus and completed in the periphery. Sequential development of thymocytes is dependent on T cell receptor (TCR) signaling and an array of transcription factors. In this study we show that special AT-rich binding protein 1 (SATB1), a T lineage-enriched chromatin organizer and regulator, is induced in response to TCR signaling during early thymocyte development. SATB1 expression profile coincides with T lineage commitment and upregulation of SATB1 correlates with positive selection of thymocytes. CD4 thymocytes exhibit a characteristic bimodal expression pattern that corresponds to immature and mature CD4 thymocytes. We also demonstrate that GATA3, the key transcriptional regulator of αβ T cells positively regulates SATB1 expression in thymocytes suggesting an important role for SATB1 during T cell development.

1. Introduction

The T lineage developmental program begins with the migration of the hematopoietic progenitors into the thymus. Thymic environment provides the necessary signals for the commitment of early progenitors towards T lineage, which then undergo an orderly process of differentiation (Starr et al., 2003). Maturing thymocytes acquire CD4 and/or CD8 coreceptors in a developmentally relevant manner. Early thymic precursors that lack either of the coreceptors are known as $CD4^-$ $CD8^-$ double negative (DN) thymocytes. This population of cells is further subdivided into four stages based on the expression of CD44 and CD25: $CD44^+$ $CD25^-$ (DN1), $CD44^+$ $CD25^+$ (DN2), $CD44^-$ $CD25^+$ (DN3) and $CD44^-$ $CD25^-$ (DN4), which bear a precursor product relationship (Lind et al., 2001). DN3 thymocytes initiate the rearrangement of T cell receptor (TCR)-β chain, which is expressed together with a non- rearranging pre-T-α chain and receive pre-TCR signals to progress to $CD4^+$ $CD8^+$ double positive (DP) stage. At the DP stage TCR-α chain locus undergoes rearrangement and the mature receptor is expressed on the cell surface. TCR-αβ+ DP thymocytes finally mature into $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ single positive (SP) thymocytes (Germain, 2002; Singer et al., 2008).

The fate of developing thymocytes is determined at multiple checkpoints. The first checkpoint is the β-selection checkpoint experienced by DN3 thymocytes that directs development to the DP stage. This checkpoint ensures the maturation of sufficient number of TCR-β expressing thymocytes that are available to rearrange and express the TCR-α chain allowing sufficient numbers of TCR-αβ expressing thymocytes to audition for selection (Singer et al., 2008). At the second checkpoint, DP thymocytes undergo positive and negative selection in response to TCR interacting with peptide-bound MHC molecules on the antigen presenting cells (Zerrahn et al., 1997). TCR signaling is critical for appropriate positive and negative selection of thymocytes to ensure appropriate tolerance and immune function both through proper positive and negative selection processes as well as the development of natural regulatory T cells (nTregs) (Starr et al., 2003). While the sequence of events during T cell development is well defined, the molecular mechanisms including the role of transcription factors are just beginning to emerge (Rothenberg et al., 2008). The role of Special AT-rich binding protein 1 (SATB1) has been studied during T cell activation (Pavan Kumar et al., 2006) and differentiation (Notani et al., 2010). SATB1 is a global regulator and chromatin organizer (Cai et al., 2003; Galande et al., 2007). In absence of SATB1 function thymocyte development is blocked at the DP stage. SATB1 null DP thymocytes ectopically express IL-2Rα and IL-7R and undergo activation induced cell death (Alvarez et al., 2000). However, the molecular mechanisms governing the role of SATB1 in T cell development remain to be determined.

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As a first step towards unraveling the role of SATB1 in T cell development and function, we monitored the expression profile of SATB1 in various thymocyte subsets and observed that SATB1 is differentially expressed. Our results further indicate that induction of SATB1 is dependent on TCR signaling and might in turn regulate the development of CD4 and CD8 thymocytes. Finally, we show that the transcription factor GATA-3 directly regulates SATB1 expression in developing thymocytes by binding to its upstream regulatory region. Collectively, this study provides mechanistic insights into the regulation of SATB1 during early T cell development.

2. Materials and methods

2.1. Mice

Age matched C57BL/6 control mice were used in all experiments. All the mice were bred and maintained according to the regulations and guidelines of National Institute of Aging, Baltimore, USA and National Centre for Cell Science (NCCS), Pune, India. The animals and procedures used in the experiments were in compliance with guidelines from the regulatory bodies of both the institutes.

2.2. Antibodies

The following fluorescent conjugated antibodies were obtained from either BD biosciences or eBioscience – CD4 (GK1.5), CD8 (53 -6.7), CD24, CD69 (H1.2F3), Qa-2, FoxP3, TCR-β (H57-597), CD44 (IM7), CD25, anti-IL-4 and anti- IFN-γ. Anti-SATB1 antibody used for ChIP and immunostaining was raised in rabbit and purified using immunoaffinity chromatography using standard procedures. FITC conjugated anti-Rabbit IgG was obtained from Boehringer Ingelheim. Anti-tubulin (T6557) and anti-β-actin (A2228) antibodies were procured from Sigma.

2.3. Cell culture

$CD4^+$ T cells were isolated from spleen and lymph nodes by negative selection using CD4 isoaltion kit (BD Biosciences). The cells were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml). $CD4^+$ T cells were treated with LY294002 (5 μM) (Calbiochem). Briefly, the cells were treated with LY294002 at indicated concentrations or vehicle control DMSO for 6 h. At the end of the time point cells were harvested and analyzed by immunoblotting.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated using RNAeasy isolation kit (Qiagen). Then total RNA (500 ng) was reverse transcribed using Dynamo cDNA synthesis Kit (Finnzymes) and oligo dT primer. Quantitative real-time PCRs were performed using SYBR green PCR master mix (Applied Biosystems), with annealing and extension of primers at 60 °C. Fold changes were calculated using the formula; Fold change = $2^{-(\mathrm{dd\ Ct})}$.

2.5. Immunoblotting

Cell lysates were prepared using RIPA lysis buffer (Tris pH 7.4, NP40 1%, Na deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM) and immunoblotting was performed as described previously (Pavan Kumar et al., 2006).

2.6. Immunostaining and FACS analysis

The cells were fixed using 2% paraformaldehyde (Sigma-Aldrich) and permeabilized using 0.1% Triton-X-100 (Sigma) followed by staining with anti-SATB1 antibody. Secondary antibody used was conjugated with Alexafluor 594. DNA counter staining was performed using DAPI. Cells were visualized under confocal microscope (Carl Zeiss). Image analysis was performed using Zen 2011 software (Carl Zeiss).

Mouse thymocytes were acquired on FACS CantoII (Becton Dickinson) and analysis was performed using Flowjo software (Tree star). Dead cells were excluded using forward and side scatter. Staining of intranuclear antigens such as SATB1 and FoxP3 was performed using Foxp3 nuclear staining kit as described by the manufacturer (eBiosciences).

2.7. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (Notani et al., 2010). Briefly, mouse thymocytes or $CD4^+$ T cells were crosslinked by 1% (v/v) formaldehyde at room temperature for 10 min and neutralized with 125 mM glycine (final) followed by washing with PBS pH 7.4. Cells were sheared using Bioruptor (Diagenode) to obtain 200–500 base pair fragments. Chromatin was incubated with specific antibodies overnight at 4 °C and respective IgG types were used as isotype controls. Protein A/G bead cocktail was then added to pulldown the antibody-bound chromatin and was subjected to elution using sodium biocarbonate buffer containing SDS and DTT (Sigma-Aldrich). Eluted chromatin was de-crosslinked and proteins were removed by digesting with proteinase K. Purified immunoprecipitated chromatin was subjected to PCR amplification using specific primers. Input chromatin was used as control.

2.8. ChIP-seq analysis

ChIP-seq analysis was performed using the data available from GEO database (GSE20898). The ChIPseq reads were aligned to the mm8 genome and peak calling on binding site was performed using MACS (Zhang et al., 2008).

3. Results

3.1. Differential expression of SATB1 in subsets of mouse thymocytes

To monitor if SATB1 is differentially expressed in various subsets of thymocytes, we sorted mouse thymocytes based on the surface expression of CD4 and CD8 coreceptors into four distinct populations – DN, DP, CD4 SP and CD8 SP and analyzed for SATB1 expression by quantitative PCR. We observed that SATB1 is minimally expressed in DN population and highly expressed in DP populations; CD4 and CD8 thymocytes have intermediate expression (Fig. 1A). Further, we monitored the expression of SATB1 at protein level by flow cytometric analysis. Mouse thymocytes were stained using lineage specific antibodies and the lineage negative subset (Lin −ve) (Fig. S1A) was analyzed for expression of CD44 and CD25 antigens to distinguish them into DN1 to DN4 stages (Fig. S1B). We then monitored the expression of SATB1 in individual DN subsets and observed that SATB1 is minimally expressed in DN1 to DN3 subsets and induced in DN4 population (Fig. 1B). These data suggested that SATB1 expression might be induced in response pre-TCR signals and facilitates β-selection checkpoint.

Next, we monitored the expression of SATB1 in DP, CD4 $CD8^{Lo}$, CD4 SP and CD8 SP thymcoyte populations and observed that expression of SATB1 in DP thymocytes was similar to the expression profile seen in DN4 thymocytes (Fig. 1B and C). However, $CD4^+$ $CD8^{Lo}$ intermediate thymocytes exhibited relatively higher expression of SATB1 as compared to DP subset. Interestingly, CD4 SP thymocytes exhibited bimodal distribution of SATB1 expression and we refer to the populations as $SATB1^{Hi}$ and $SATB1^{Lo}$CD4