RNA editing enzyme ADAR1 is required for early T cell development

Richard Xufeng, Daibang Nie, Qiong Yang, Wang Wang, Tao Cheng, Qingde Wang

Abstract
The RNA editing enzyme ADAR1 has been shown to be an essential molecule for hematopoietic cell differentiation, embryonic development, and regulation of immune responses. Here, we present evidence in a T-cell-specific gene knockout mouse model that ADAR1 is required for early T cell development. Loss of ADAR1 led to cell death of the progenitors at the double negative stage and prevented T cell maturation in the thymus. Furthermore, ADAR1 deletion in pre-T cells preferentially affected TCRβ-expressing cells causing TCRβ positive cell depletion. Interruption of IFN signaling occurred in the premature T cells, indicating a role of IFN signaling in the survival of TCRβ-expressing cells regulated by ADAR1. This study demonstrated an essential role for the RNA editing enzyme ADAR1 as a potential regulator for T-cell fate determination during clonal selection, which, in turn, contributes to immunologic homeostasis.

Keywords: ADAR1, Conditional knockout, RNA editing, T cell development

1. INTRODUCTION
The mechanisms of self-tolerance by the immune system and the clonal selection of functional lymphocytes are central concerns of the immunology society. Lymphoid progenitors arise from bone marrow stem cells and migrate into thymus, where they interact with the cortical and medullar thymic epithelial cells to differentiate into mature T cells. From the progenitor to mature T cell stage, thymocytes have been phenotypically characterized to distinct stages, defined as double negative (DN), double positive (DP), and single positive (SP) stages, featuring the expression of differentiation antigens CD4 and CD8. DN cells are further categorized into DN1, DN2, DN3, and DN4 subpopulations, based on the transient expression of CD44 and CD25. In such ordered process of T cell development, T cell receptor (TCR) expression is one of the most critical events. TCRβ recombination begins at the DN2 stage and continues to the DN3 stage. Functional TCRβ is expressed on the surface of DN3 cells, where it interacts with pre-T cell receptor α (pre-TCRα) and CD3 to form the pre-TCR, which is a critical mediator of cell proliferation, survival, and further maturation. At the DP stage, TCRs undergoes VDJ recombination and both TCRα and TCRβ are expressed to form the cell surface TCRαβ receptors. Functional T cell clones subsequently undergo the positive and negative selection and lineage commitment of CD4 or CD8 SP cells. The selection mechanism determining cell fate is yet to be fully understood.

Adenosine deaminase acting on RNA-1 (ADAR1) is an RNA editing enzyme and plays an essential role in embryonic hematopoiesis. Through its deamination activity, ADAR1 converts adenosine to inosine in RNA molecules (A-to-I RNA editing). Inosine is equivalent to guanosine in Watson–Crick structure and during protein translation. A-to-I RNA editing therefore durably modifies sequences of targeted RNAs and can alter proteins that they encode. Proteins coded by edited RNAs, such as GluR-B and SR2C, exhibit dramatically different properties compared with their genome-coded forms. In addition, RNA splicing sites can be generated or eliminated by this A-to-I conversion. For example, once edited, some microRNA precursors modulate the efficacy of mature microRNA genesis or shift targets. In addition, ADAR1 has been proposed to participate in the processes of RNA transportation and degradation and to regulate gene expression, through
protein–protein interactions. We recently reported that RNA editing activity of ADAR1 was required for the differentiation of adult hematopoietic progenitor cells. In order to explore the function of ADAR1 in T cells, we generated a mouse model in which ADAR1 was specifically deleted in the early stage of T cell differentiation. Our data have demonstrated an essential role of ADAR1 in T cell development, which was associated with TCRβ expression.

2. RESULTS

2.1. Dramatic reduction of mature T lymphocyte in ADAR1-deficient mice

By crossing ADAR1<sub>lox/lox</sub> and Lck-Cre transgenic mice, a conditional knockout mouse strain was generated (genotype: ADAR1<sup>lox/lox</sup> and Lck-Cre<sup>+</sup>, referred to as ThyA1<sup>d</sup> hereafter), in which the ADAR1 gene was deleted by Cre-mediated recombination specifically in T cells (Fig. 1A). ADAR1 deletion in these cells was confirmed by genotypic analysis with FACS-purified lymphocytes and tissue DNA samples (Fig. 1B). ThyA1<sup>d</sup> mice were healthy, reared under pathogen-free conditions, and showed no difference in appearance from their control (Lox/Lox or Wild/Wild-Cre<sup>+</sup>) littermates. There was a dramatic reduction in the number of T lymphocytes in the peripheral blood of most ThyA1<sup>d</sup> mice in comparison to the controls (Fig. 1C). Whereas the total numbers of DP and SP cells were dramatically diminished, the total number of DN cells was unaffected by ADAR1 deletion (Fig. 1E). ADAR1 was therefore required either for the transition from DN to DP, for survival through DP, or for both processes during T cell development. To confirm the specific impact of ADAR1 on DN, DP, and SP cells, we verified the ADAR1 gene deletions in these cells by semiquantitative PCR as described previously. The ADAR1 gene was almost completely deleted in DP cells, but majorities of DN and SP cells had an intact ADAR1 gene (Sup. Fig. 2B, http://links.lww.com/BS/A9).

2.2. Blockage of T cell development in the thymus

Thymuses of ThyA1<sup>d</sup> mice appeared grossly smaller than the controls (Fig. 2A). Pathological studies revealed dramatic cell depletion. As was evident in H-E stained sections, the tissue architecture was severely disrupted and a pronounced scarcity of lymphocytes was observed, especially in the cortex regions (Sup. Fig. 2A, http://links.lww.com/BS/A9). Consequently, the total cellularity of the thymus was dramatically lower than that of their littermates (Fig. 2B). To identify the putative defect of T cell development in the thymus corresponding with the morphological abnormalities, phenotypic markers of CD4 and CD8 on the thymocytes were analyzed by flow cytometry. A marked decrease in the percentage of DP cells and an increase in DN cells were observed (Fig. 2C and D). Whereas the total numbers of DP and SP cells were unaffected by ADAR1 deletion (Fig. 2E), ADAR1 was therefore required either for the transition from DN to DP, for survival through DP, or for both processes during T cell development. To confirm the specific impact of ADAR1 on DN, DP, and SP cells, we verified the ADAR1 gene deletions in these cells by semiquantitative PCR as described previously."
death indicated by DAPI staining was observed in the DP and the SP, but not the DN cells (Fig. 2F and Sup. Fig. 2C, http://links.lww.com/BS/A9). These findings support a DN4-stage-specific requirement of ADAR1 during thymocyte differentiation.

2.3. ADAR1 deficiency impacts T cell development at DN4 stage

In ThyA1d mice, the total number of DN cells was not significantly different from that of the controls, mostly due to the relatively moderate ADAR1 deletions in these cells (Fig. 2F and Sup. Fig. 2B, http://links.lww.com/BS/A9). Given that the recombination of ADAR1 loci was mediated by Cre-recombinase that was expressed under the control of the lymphocyte-specific kinase (Lck) promoter, ADAR1 gene deletion might have occurred in only a subpopulation of the DN cells in ThyA1d mice. Lck is active from DN1 to DN4 stages and its activity dramatically (>20 times) increases in DN3 cells. However, other studies indicated that the Lck-Cre transgene only yielded 50% deletion of a floxed gene in DN4 cells and was more inefficient than earlier indicated in DN3 cells.24 We therefore decided to determine at which DN stage ADAR1 alleles started to be efficiently deleted in ThyA1d mice. DN1–DN4 cells were fractionated by the combination of CD44 and CD25 markers from individual ThyA1d and heterozygous mice (Sup. Fig. 3, http://links.lww.com/BS/A9). Relative quantities of the floxed and deleted alleles were analyzed with the sorted cells. The results showed that the floxed ADAR1 gene could be deleted in, as early as, the DN1 stage in the heterozygous mice (Wild/Lox-Cre+, sup panel in Fig. 3A). In homozygous (Lox/Lox-Cre+) cells, the deletion became efficient from the DN2 stage and markedly increased in DN3 and DN4 cells. We also noticed the individual variations of the recombination efficiency among different mice.

Only the mice with greater than 80% ADAR1 deletion in DN3 and DN4 were used for further analysis. Interestingly, both percentages and total cell number of DN4 cells were significantly decreased, in contrast to the unchanged or increased numbers in the DN1 to DN3 cells (Fig. 2B and C). While ADAR1 alleles were deleted efficiently in both DN3 and DN4 cells, only the DN4 cell population, but not the DN3 cells, was decreased significantly in ThyA1d mice. These findings support a DN4-stage-specific requirement of ADAR1 during thymocyte differentiation.

2.4. ADAR1 deficiency is associated with TCRβ positive cells

To determine if ADAR1 was involved in a particular molecular event critical for T cell development, we examined whether TCR expression was affected in the ThyA1d mice. Because TCRβ locus recombination occurs at DN2 and DN3 stages and functional TCRβ is expressed on the surfaces of a significant portion of DN4 cells, we examined whether the cell surface expression of TCR was affected in the absence of ADAR1 on DN4 cells. Both TCRβ and TCRβ/γ antibodies were used for flow cytometry staining. The percentage of TCRβ+ cells were found to be dramatically decreased in ThyA1d mice, whereas TCRβ/γ+ cells were the opposite (Fig. 4A and B, Sup. Fig. 4A-B, http://links.lww.com/BS/A9), which indicated that ADAR1 deletion preferentially affected TCRβ+ cells. The preferential suppression of TCRβ+ cells was also observed in more mature cells in ThyA1d mice (Fig. 4C). ADAR1 alleles were almost completely deleted in both TCRβ+ and TCRβ- cells at DN4 and DP stages, while the deletion was not efficient in TCRβ/γ+ cells (Sup. Fig. 4C, http://links.lww.com/BS/A9). This may also account for the increased percentage of the TCRβ/γ+ cells in the DN4 population of ThyA1d mice. On the other hand, ADAR1 gene was efficiently deleted in both TCRβ+ and TCRβ- cell populations; the TCRβ+ cells can barely survive in the absence of ADAR1, in contrast to the TCR- cells that stayed intact.

Figure 2. T cell development was severely blocked in the thymus of ThyA1d mice. (A) Thymuses of ThyA1d mice were severely hypogenetic. The size was markedly smaller than the controls. (B) The total cell numbers of thymocytes were shown, data indicate the mean ± SEM from the mice of three independent litters. (C) Thymocytes were phenotypically analyzed with CD4 and CD8 antigen expressions. (D) The percentages of DN, DP, and SP thymocytes within CD4+ cells were shown, n = 16 (ADAR1 Lox/Lox-Cre+) and n = 14 (Lox/Lox). (E) The absolute numbers of each subpopulation were shown, n = 6 (ADAR1 Lox/Lox-Cre+) and n = 7 (Lox/Lox). (F) Massive cell death was detected in the DP and SP cells in ThyA1d mice monitored by DAPI staining. n = 6 (ADAR1 Lox/Lox-Cre+) and n = 7. All the data indicate the mean ± SEM. *P < .05, **P < .01, and ***P < .001 (t test).
2.5. Elevated expression of IFN target genes in ADAR1-deficient thymocytes

ADAR1 suppresses IFN signaling in hematopoietic cells, which has been proposed to underlie an ADAR1 requirement in progenitor cell survival.\textsuperscript{26} We therefore examined whether disrupted IFN signaling occurred in ThyA1d mice. A subset of IFN-regulated genes was selected for assay in ThyA1d and control thymocytes. These included Stat-1, interferon-regulated factor 9 (IRF-9), interferon-induced protein 35 (IIP-35), and double-stranded RNA-dependent serine/threonine protein kinase (PKR, or EIF2AK2). Expressions of all these genes showed marked increase in ADAR1-deficient hematopoietic cells.\textsuperscript{26} Additionally, the expressions of these genes were also observed in ThyA1d thymocytes. Meanwhile, PKR and the IFN-inducible gene, interferon-inducible GTPase 1 (lpg1), were both significantly upregulated (Fig. 4D). Although the increases of Stat-1, IRF-9, PKR, and IIP-35 were less dramatic than reported in hematopoietic stem/progenitor cells, it was evident that loss of IFN signaling suppression could also characterize ADAR1-deficient T cells in the ThyA1d thymuses.

3. DISCUSSION

Functions of ADAR1 have been well established in hematopoiesis, especially in hematopoietic progenitors.\textsuperscript{22,26} Yet the roles of ADAR1 in the development of lymphocytes remain to be determined. Here, we demonstrated that ADAR1 plays an essential role during T cell development to support the survival of T cell progenitors in the thymus. With ADAR1 deficiency, massive cell death happened in the thymus in the early stages of T cell progenitors. Our data indicated that ADAR1 is essential for T cell development from DN4 stage onward. An ADAR1 requirement in DN1 and DN2 T cell progenitors cannot be discounted, but insufficient ADAR1 deletion in our Lck-Cre mice at DN1 and DN2 stages prevented the testing of this possibility.

It has been demonstrated that loss of ADAR1 leads to cell death in particular cell types that undergo differentiation at certain stages. In the marrow, ADAR1 is dispensable for the hematopoietic stem cells, but the differentiating progenitors cannot survive without ADAR1.\textsuperscript{22,26} In mouse embryos, normal development is not interrupted by ADAR1 deficiency during the first half gestation, but cell death happens in multiple cell types when they are in embryonic day 11–11.5.\textsuperscript{11} In the thymus, TCR\textsubscript{β} recombination initiates at DN2 cells and continues to DN3 cells.\textsuperscript{3} A functional impact of ADAR1 deletion first appeared in DN4 cells where TCR\textsubscript{β} starts to be expressed on the cell surface. TCR\textsubscript{β} expression provides signals for allelic excision, proliferation, survival, and further maturation.\textsuperscript{9} One of the most striking findings of this study is the association of ADAR1 deficiency and the diminishing of TCR\textsubscript{β} expression, but it is currently unknown how the loss of ADAR1 specifically reduce TCR\textsubscript{β}+ cells. However, our data suggest that the contribution of proper TCR expression to thymocyte ontogeny can be disrupted in the absence of ADAR1.
ADAR1 is an RNA editing enzyme and the mechanism of nucleotide conversion has been well studied. The mechanism of cell death that arises from ADAR1 deficiency, nevertheless, is barely understood. No particular RNA target has been identified related to cell death. Recently, it has been reported that ADAR1 suppresses interferon signaling and its deletion in hematopoietic cells upregulated gene expressions downstream of interferon. IFN signaling has been shown centrally involved in lymphocyte development. In T cells, ADAR1-mediated suppression of type I interferon-stimulated gene expression is required for thymic T cell self-tolerance and prevention of colitis. Accordingly, we observed significantly increased expression of IFN-regulated genes, especially PKR and Irg1, in the thymocytes of Thy1d mice. Elevated PKR enhances the phosphorylation of translation initiation factor eIF-2a and suppresses protein translation, and the absence of ADAR1 will further accelerate its kinase activity in addition to the higher level of PKR. Deregulated expression of interferon-responsive genes could contribute to the cell death phenotype in the Thy1d mice.

In T cell progenitors, ADAR1 deficiency switches cell fate to death, which is triggered by TCR expression. Elaboration of the molecular mechanisms of ADAR1 in cell fate determination after TCR expression will likely lead to a better understanding of cell survival under the positive or negative clonal selection during T cell development.

4. MATERIALS AND METHODS

4.1. Mice

The ADAR1lox/lox mice were generated as described in Ref. [11]. B6.Cg-Tg (Lck-Cre) 548Jxm/J mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and crossed with our ADAR1lox/lox mice in the animal facility of University of Pittsburgh Cancer Institute with IACUC approval.

4.2. Genotyping

DNA samples from mouse tail tip or fractionated lymphocyte subpopulations were analyzed by semi-quantitative PCR with multiple primers as described previously. For the floxed, deleted, and wild-type ADAR1 alleles, we use the commonsense primer competitively for their amplification in PCR reaction and the ratio of the products represent the relative quantities of the corresponding ADAR1 alleles.

4.3. Preparation of thymocytes, splenocytes, lymphonode lymphocytes, and peripheral blood cells

Thymus, spleen, and lymphonode were collected from 4–12-week-old mice and minced into small pieces in the presence of HBSS (Lonza, Allendale, NJ) supplied with 2% FBS. A single cell suspension was prepared using 40 μm cell strainer (BD, San Jose, CA). Excessive red blood cells were removed by incubation with...
ACK lysing buffer (Lonza) per manufacturer’s instruction. The peripheral blood was sampled from tail vein bleeding and peripheral blood mononuclear cells were prepared using ACK lysing buffer. All cells were washed with HBSS before staining for flow cytometry analysis. Cellularity was counted using AcT10 hematology analyzer (Beckman Coulter, Miami, FL).

4.4. Flow cytometry and cell sorting

All antibodies used for flow cytometry were purchased from eBioscience (San Diego, CA) unless otherwise mentioned. Peripheral blood mononuclear cells, splenocytes, and lymphnode lymphocytes were stained with CD45, CD3, CD4, CD8, B220 (BD, San Jose, CA), and CD11b (Gr-1). The thymocytes were stained with CD45, CD4, CD8, CD25, CD44, TCRβ, and TCRα/β. All the surface markers were examined by Cyan Cytometer (Dako, Carpantaria, CA) and the FACS cell sorting was performed by MoFlo (Dako) or Aria II (BD).

4.5. Histology

Tissues were fixed with 10% buffered formalin overnight and the samples were processed and analyzed in the Department of Pathology in University of Pittsburgh Cancer Institute.

4.6. Real-time RT-PCR

RNA was extracted from thymocytes using the RNA STAT-60 kit (TEL-TEST Inc.) according to the manufacturer’s instructions. Reverse transcription was carried out using random hexamer oligonucleotide primers and Super-Script II (Invitrogen) reverse transcriptase for 15–30 minutes. Quantitative real-time PCR analyses were performed in triplicate, using BioFast EvaGreen PCR Supermix (Bio-Rad) and gene-specific primers (Sup. Table 1, http://links.lww.com/BS/A9). The expression levels were calculated according to the standard curves and were normalized by those of β-actin, HPRT, or GAPDH. The data were presented as relative fold change.

4.7. Statistics

All comparisons between experimental groups and the control groups were done by student t-test, unless otherwise mentioned. *, P < 0.05; **, P < 0.01, and ***, P < 0.001.

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