



## Modeling Sjögren's syndrome with *Id3* conditional knockout mice

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### ABSTRACT

The *Id3* gene has been shown to play important roles in the development and function of broad tissue types including B and T cells. *Id3* deficient mice develop autoimmune disease similar to human Sjögren's syndrome. Both B and T lymphocytes have been implicated to contribute to the disease phenotype in this disease model. In order to gain a better understanding of individual cell types in this disease model, we generated an *Id3* conditional allele. An *LckCre* transgene was used to induce *Id3* deletion in developing T cells. We showed that the *Id3* gene was efficiently disrupted in early thymocyte development prior to T cell receptor (TCR)-mediated positive selection. Consequently, thymocyte maturation was impaired in the conditional knockout mice. These mice developed exocrinopathy starting at two months of age and subsequently exhibited high incidence of lymphocyte infiltration to salivary glands between eight and 12 months of age. This progressive feature of disease development is very similar to those observed in *Id3* germline knockout mice. This study establishes a new model for investigating the relationship between T cell development and autoimmune disease. Our observation provides an experimental case that autoimmune disease may be induced by acquired mutation in developing T cells.

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### 1. Introduction

T cell development occurs in the thymus where a diverse repertoire of T cell receptors (TCR) is generated and clonally selected. The expression of either  $\alpha\beta$ TCR or  $\gamma\delta$ TCR heterodimers results in developmentally and functionally distinct  $\alpha\beta$  or  $\gamma\delta$ T cell population, respectively. While a small fraction of thymocytes develop to  $\gamma\delta$ T cells, the majority of thymocytes enters the  $\alpha\beta$ T cell fate and undergoes further selection and lineage differentiation. These freshly produced  $\alpha\beta$ TCR positive thymocytes also express the CD4 and CD8 co-receptors, and thus named CD4CD8 double positive (DP) cells. The DP thymocytes undergo a rigorous selection, which eliminates more than 95% of the population and allows the remaining ones to become either CD4 helper or CD8 cytotoxic single positive (SP) T cells [1,2]. This selection process is critical for T cells to acquire the ability of providing antigen specific immune function while maintaining tolerance to self-antigens.

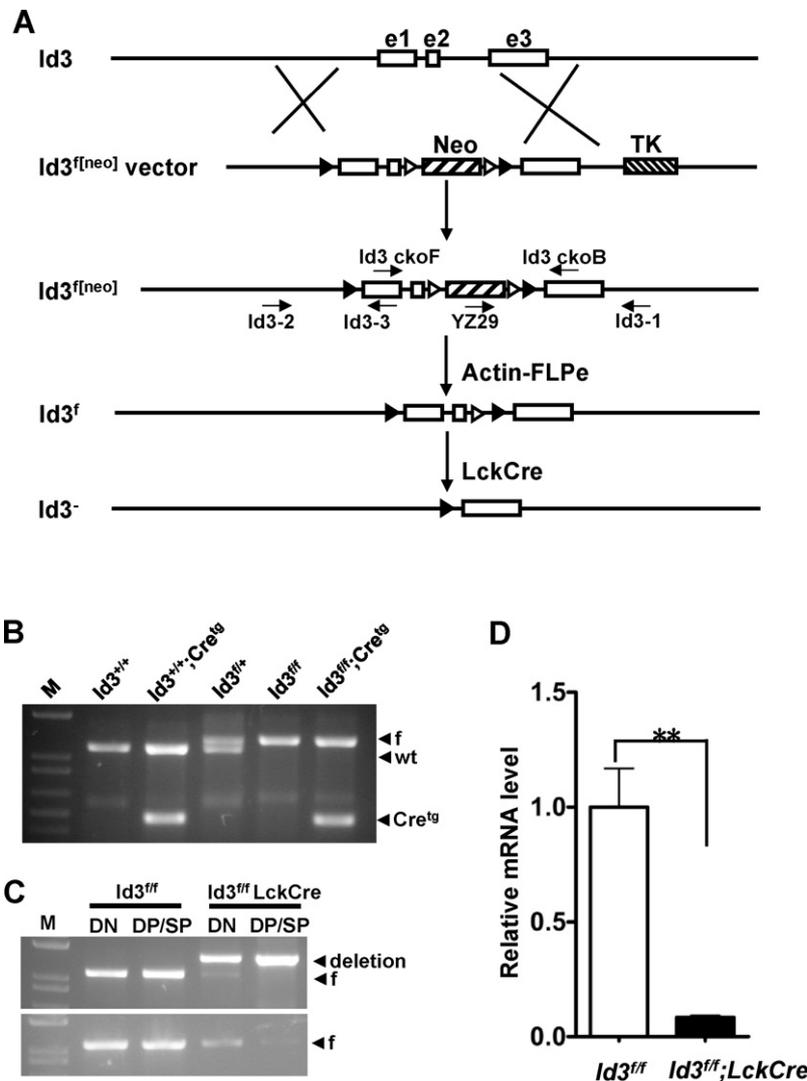
*Id3* is known to play important roles in mediating TCR signals during DP to SP selection [3]. The *Id3* gene encodes a 13 kDa helix–loop–helix protein which primarily functions as a competitive inhibitor of E-protein transcription factors such as E2A and HEB [4–6]. *Id3* was first isolated from cultured fibroblasts as an immediate early gene responding to serum stimulation [7]. It has been confirmed in many tissue types that *Id3* up-regulation promotes cell proliferation partially through inhibition of E-protein activities. Although *Id3* is expressed in broad tissue types, *Id3* seems to play a particularly important role in the lymphoid system. *Id3* germline knockout (*Id3*<sup>−/−</sup>) mice exhibit multiple defects in both B and T cell compartments. Humoral immune responses to T cell dependent antigens and type 2 T cell independent antigens were impaired in *Id3*<sup>−/−</sup> mice [8]. B cell proliferation upon BCR cross-linking by IgM treatment was also perturbed in *Id3*<sup>−/−</sup> mice. Analysis of *Id3*<sup>−/−</sup> mice also revealed a reduction of both CD4 and CD8 single positive T cell population and an enhancement of  $\gamma\delta$ T cell population in the thymus [9,10]. Introduction of the *AND* or *HY* transgenic TCR into the *Id3*<sup>−/−</sup> background confirmed a necessary role for *Id3* in both positive selection and negative selection [10].

In addition to the developmental defects, *Id3*<sup>−/−</sup> mice develop primary Sjögren's syndrome-like symptoms [11]. Sjögren's syndrome is a common rheumatic autoimmune disease with a prevalence of about 0.6% in the general population [12–14]. The main clinical features are persistent dry eyes and mouth, pathological score of focal infiltrates in salivary gland biopsy, and serum positive for multiple autoantibodies such as anti-Ro and anti-La.

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**Fig. 1.** Conditional deletion of the *Id3* gene in developing thymocytes. (A) Generation of the *Id3* conditional deletion allele. Open box: exon. Hatch box: selection markers. Solid arrowhead: loxP. Open arrowhead: FRT. Arrows: primers. (B) Representative genotyping results of the *Id3<sup>f/f</sup>* allele. DNA samples were prepared from mouse toes. 4 primers were used: Id3-2 and Id3-3 for the loxP (1.25 kb) and the wild type (1.15 kb) *Id3* allele, Tek-F and Tek-R for the *LckCre* transgene (0.48 kb). M: 1 kb plus DNA ladder. (C) Deletion of *Id3* in thymocytes sorted from 7-week-old mice. Thymocytes were separated into the DN ( $CD4^-CD8^-$ ) and DP/SP ( $CD4^+CD8^-$ ,  $CD4^+CD8^+$ , and  $CD4^-CD8^+$ ) groups by magnetic beads. For the upper panel, the floxed and deletion alleles were determined by competitive PCR using three primers, Id3-2, Id3ckoF and Id3ckoB shown in (A). It produced a 1.07-kb band for the *Id3<sup>f</sup>* allele and a 1.34-kb band for the deleted allele. For the bottom panel, only two primers (Id3ckoF and Id3ckoB) were used to detect the floxed allele. (D) Real-time quantitative PCR analysis of *Id3* expression. RNA was prepared from total thymocytes of 7-week old *Id3<sup>f/f</sup>* (open bar,  $n=4$ ) and *Id3<sup>f/f</sup>;LckCre* (filled bar,  $n=3$ ) mice.  $**P=0.006$ . The graphed results are means with SEM.

*Id3*<sup>-/-</sup> mice were found to exhibit a reduction of tear and saliva as early as two months of age, prior to any detectable sign of lymphocyte infiltration in the gland tissues. Lymphocyte infiltration in salivary and lachrymal glands can be detected at a later time around six months of age. Anti-Ro and anti-La auto-antibodies were detected in two thirds of mice after they have reached one year of age. This study also identified a T cell intrinsic role for *Id3* contributing to disease development. However, depletion of B cells in *Id3*<sup>-/-</sup> mice by CD20 antibody treatment could also ameliorate the disease symptoms, indicating B cell involvement in disease pathology [15]. Given the broad expression pattern of *Id3* in development and in most somatic tissues, it remains to be determined unambiguously whether loss function of *Id3* in T cells accounts for the initial trigger of the autoimmune symptoms observed in *Id3* knockout mice.

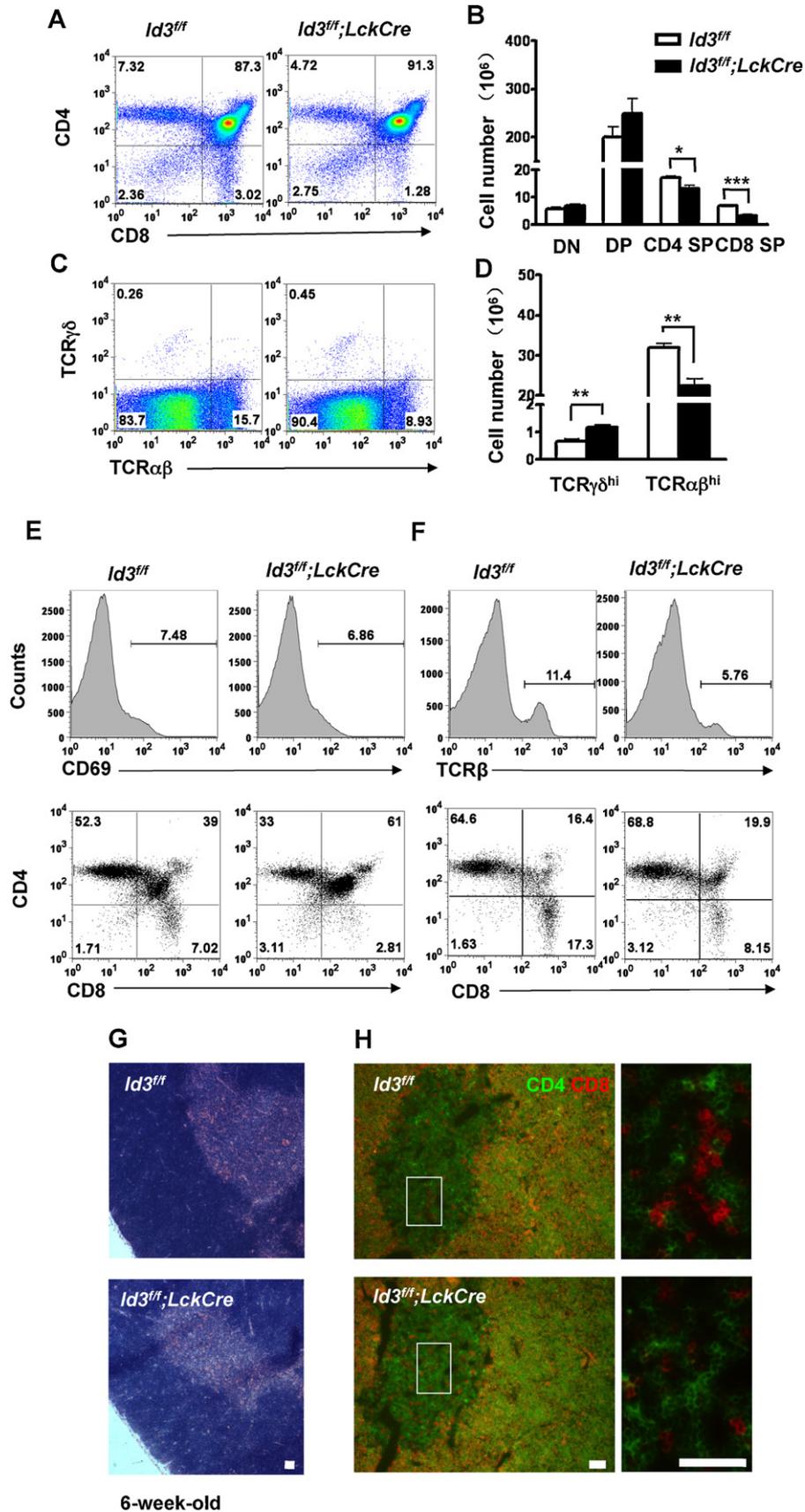
A recent study of human *Id3* gene revealed no evidence of any germline mutations or unique polymorphisms in over 200 patients clinically diagnosed with primary Sjögren's syndrome [16]. However, this study is not sufficient to rule out the possibility that

acquired mutations or epigenetic changes in the lymphoid lineage may play a greater role than germline mutations in human patients. It is not known whether loss function of *Id3* in lymphocytes could have played a primary role in the development of Sjögren's syndrome. To further understand the role of *Id3* in lymphocyte development and immune tolerance, we developed an *Id3* floxed allele for conditional removal of *Id3* in specific cell lineage. Studies presented here demonstrated that conditional disruption of *Id3* in the developing T cells leads to impaired thymocyte selection and development of autoimmune symptoms similar to Sjögren's syndrome.

## 2. Materials and methods

### 2.1. Generation of *Id3* conditional deletion allele

The targeting vector is composed of a 5.7 kb long arm and a 2.5 kb short arm sequences from the *Id3* genomic sequence. The 5' and 3'



**Fig. 2.** Disturbed  $\gamma\delta$ T and  $\alpha\beta$ T cell development in the thymus of *Id3<sup>fl/fl</sup>;LckCre* mice. (A) Representative FACS analysis of  $\alpha\beta$  thymocyte development by CD4 and CD8 expression. Thymocytes were isolated from 4 to 6 weeks old *Id3<sup>fl/fl</sup>;LckCre* ( $n=3$ ) and the *Id3<sup>fl/fl</sup>* littermate ( $n=4$ ) controls. Dead cells were excluded by 7AAD staining. (B) Summary of the absolute numbers of DN ( $CD4^+CD8^-$ ), DP ( $CD4^+CD8^+$ ), CD4 SP ( $CD4^+CD8^-$ ), and CD8 SP ( $CD4^-CD8^+$ ) thymocytes. \* $P=0.027$ . \*\*\* $P=0.0001$ . The statistical significance was assessed by unpaired student's  $t$  test. (C) Representative FACS plots of  $\gamma\delta$  and  $\alpha\beta$  thymocyte population in *Id3<sup>fl/fl</sup>;LckCre* mice and *Id3<sup>fl/fl</sup>* mice. (D) Summary of the absolute numbers of mature  $\gamma\delta$  and  $\alpha\beta$  thymocytes. \*\* $P=0.009$  ( $\gamma\delta$ T cells) and 0.003 ( $\alpha\beta$ T cells). The graphed results are means with SEM. (E) The CD69<sup>hi</sup> cells in total

loxP sequences were inserted upstream of the first exon and in the second intron, respectively. P<sub>gk</sub>-Neo and P<sub>gk</sub>-TK were used as the positive and negative selection marker, respectively (Fig. 1A). The linearized targeting vector was introduced into W4/129S6 embryonic stem cells (Taconic). Genotyping PCR screen with primers Id3-1 and YZ29 (Table S1) identified 14 positive clones from 94 G418-resistant ES clones. These positive clones were subsequently confirmed by Southern analysis and their qualities were verified by karyotyping. Germ line transmission of the targeting allele was obtained from two independent ES clones. The P<sub>gk</sub>-Neo cassette was then deleted by crossing the positive offspring to *Actin-FLPe* mice [17]. The resulting *Id3* floxed allele (labeled *Id3<sup>f</sup>*) was crossed with *LckCre* transgenic mice for T lineage specific *Id3* deletion [18]. Primer pairs Id3-2/Id3-3, Tek-F/Tek-B, and Flp-F/Flp-B were used for the genotyping PCR of Id3 loxP sites, Cre, and FLPe, respectively (Table S1). The details of genotyping PCR were described previously [19]. Mice were bred and maintained in the mouse facility of the Institute of Developmental Biology and Molecular Medicine following the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and institutional regulations.

## 2.2. Antibody staining, FACS analysis and cell sorting

Single cell suspension from thymus, spleen and lymph node samples was prepared in PBS with 5% bovine calf serum. Approximately 10<sup>6</sup> cells were used for antibody staining. FACS antibodies include FITC-conjugated anti-CD4 (Caltag, 83-RM2501-1), PE-conjugated anti-CD8b (Caltag, 83-RM510-3), APC-conjugated anti-TCR $\beta$  (Caltag, 83-HM3605), FITC-conjugated anti-TCR $\gamma\delta$  (Caltag, 83-HM3801), FITC-conjugated CD69 (Caltag, 83-HM4001), PE-conjugated anti-TCR $\beta$  (eBioscience, 85-12-5961-81), PE-conjugated CD44 (Caltag, 83-RM5704), PE-conjugated CCR7 (eBioscience, 85-12-1971-82), APC-conjugated anti-CD8b (Caltag, RM-5015), APC-conjugated anti-CD25 (Caltag, 83-RM6005), APC-conjugated CD62L (eBioscience, 17-0621), and APC-conjugated anti-B220 (Caltag, 83-RM2605). Seven-amino-actinomycin D (7AAD) (Invitrogen, A1310) was used to exclude dead cells. For CCR7 staining, the binding of CCR7 was in 37 °C water bath for 30 min. Co-staining with other antibodies was carried out at 4 °C for 30 min. FACS analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). For cell sorting, about 10<sup>7</sup> cells were stained by rat anti mouse CD4 and CD8 antibodies, then washed and resuspended. Magnetic beads conjugated-anti rat antibody (Invitrogen Cat. #. 110.35) was applied to the cell suspension for separation of DN cells from DP/SP cells.

## 2.3. Histological and pathological analysis

Tissues were embedded in OCT, frozen in liquid nitrogen-cooled isopentane. Slides of 8- $\mu$ m tissue sections were stained according to the standard H&E staining protocol. Images were analyzed by a Leica DM RXA2 microscope, and photographs were taken by a Leica DC350F camera. Infiltration number was scored using the method described previously [11]. Basically, one infiltrate is defined as 50 or more nucleated cells in the cluster. The score for each mouse is the total infiltrates present in two histological sections prepared from one facial side including one parotid gland and one submandibular gland.

## 2.4. Quantitative real-time RT-PCR

Total RNA was prepared with TRIzol (Invitrogen, Cat#. 15596-018). DNase I treatment and reverse transcription were carried out with TaKaRa RNA PCR Kit (AMV) according to the manufacturer's instructions. Quantitative RT-PCR was performed and analyzed on an Mx3000P Quantitative PCR System (Stratagene) using the Absolute QPCR SYBR Green Mixes (ABgene, Cat#. AB-1166/b) according to the standard protocol. The primers of *Id3* were *mId3QPCR* and *mId3QCRB*. The primers for the  $\beta$ -actin internal control were *Actb-L1* and *Actb-R1* (Table S1).

## 2.5. Saliva secretion test

Saliva secretion test was performed according to the procedures previously described [11]. In brief, mice were anesthetized by intraperitoneal injection of Avertin (Sigma Cat#. T4, 840-2). Pilocarpine hydrochloride (Sigma, Cat#. P-6503) was administered to mice intraperitoneally with 0.5  $\mu$ g/g of body weight. Saliva was collected for 9 min and measured using 100- $\mu$ l sized microcapillary pipet (VWR, Cat#. 53432-921). The volume of saliva was normalized by body weight.

## 2.6. Statistical analysis

Statistical significance was determined by the unpaired student's *t*-test.

## 3. Results

### 3.1. Establishing the *Id3* conditional knockout mice

Mice carrying a germline mutation in the *Id3* gene develop autoimmune symptoms similar to human primary Sjögren's syndrome [11]. This study also indicated a dominant role for *Id3* deficient T cells in disease development. However, the study of T cell function is complicated by the fact that functions of other cells such as B cells are also affected by *Id3* disruption. To further investigate the lineage and stage specific function of *Id3* in this disease model, we generated a floxed *Id3* allele using Cre-loxP and FRT-FLPe systems. Briefly, two loxP sequences were introduced into the *Id3* locus flanking the first two exons, which contain the entire coding region of the Id3 protein. A P<sub>gk</sub>-Neo expression cassette flanked by two FRTs was inserted in the second intron to provide a positive selection during transfection of ES cells (Fig. 1A). After successful germ-line transmission of the targeted ES clones, the P<sub>gk</sub>-Neo cassette was deleted by Flp/FRT mediated recombination in mice expressing an *Actin-Flpe* transgene [17]. The resulting *Id3<sup>f</sup>* strain carried the *Id3* floxed allele to be used for subsequent conditional knockout studies. *Id3<sup>ff</sup>* homozygous mice were viable and phenotypically indistinguishable from wild type or heterozygous littermates.

To delete the *Id3* gene specifically in developing T cells, we crossed the *Id3<sup>f</sup>* mice with *LckCre* transgenic mice [20]. The earliest activation phase for the *LckCre* transgene was mapped to the DN2 stage and the expression of Cre was restricted within T cell lineage [18]. The *Id3<sup>ff</sup>* mice were used as controls in the following experiments. The primer pair Id3-2 and Id3-3 was used to detect the 5' loxP site and the primer pair Tek-F and Tek-R was used to detect the *LckCre* transgene (Table S1). These primers were used in the same

thymocytes were gated for two-D plot analysis of CD4 and CD8 expression. (F) The TCR $\beta$ <sup>hi</sup> thymocytes were gated for the analysis of CD4 and CD8 expression. Results of (E) and (F) were representatives of four littermate pairs. (G) H&E staining of thymus section from *Id3<sup>ff</sup>;LckCre* mice and *Id3<sup>ff</sup>* mice. (H) Two-color immunofluorescence analysis of thymus section with CD4 and CD8 antibodies. The green and red signals are CD4 and CD8 SP thymocytes, respectively. The yellow signals are CD4<sup>+</sup>CD8<sup>+</sup> DP thymocytes resulting from CD4 and CD8 double staining. The enlarged views of medulla are shown on the right. The scale bar equals 50  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

PCR to yield a 1.25 kb and a 1.15 kb product representing the *Id3<sup>fl</sup>* and the wild type allele, respectively. The PCR product of the *LckCre* transgene was 0.48 kb (Fig. 1B). To examine the efficiency of *LckCre* induced deletion of *Id3*, we designed a three-primer competitive PCR assay (*Id3ckoF*, *Id3ckoB* and *Id3-2*) to reveal both the floxed and the deleted allele simultaneously. PCR test of total thymocytes isolated from *Id3<sup>fl/fl</sup>* and *Id3<sup>fl/fl</sup>;LckCre* mice showed exclusively the floxed band and the deletion band, respectively (Fig. S1). A mix of equal amount of thymocyte DNA from *Id3<sup>fl/fl</sup>;LckCre* mice and *Id3<sup>fl/fl</sup>* mice resulted in approximately equal amount of the deletion and floxed bands in the three-primer reaction (Fig. S1). Thus, the two alleles were detected with similar efficiency in this PCR test. Thymocytes from *Id3<sup>fl/fl</sup>;LckCre* mice and *Id3<sup>fl/fl</sup>* mice were separated into DN ( $CD4^-CD8^-$ ) and DP/SP ( $CD4^+CD8^+$ ,  $CD4^+CD8^-$ , and  $CD4^-CD8^+$ ) fractions by Dynal bead sorting. DN cells were enriched from less than 5% to more than 90% after bead depletion (Supplemental Fig. 1B). Using the three-primer PCR test, we found that deletion efficiency increased as thymocytes mature from the DN stage to the DP/SP stage (Fig. 1C, upper panel). PCR with primer pairs exclusive for the floxed allele further confirmed a substantial loss of the floxed allele among the DP/SP cells in the presence of the *LckCre* transgene (Fig. 1C, bottom panel). Because DP cells represent approximately 80% of the DP/SP fraction, we concluded that the deletion of *Id3* was completed in a large fraction of cells reaching the DP stage. To verify the inactivation of *Id3* at the transcriptional level, mRNA from total thymocytes of *Id3<sup>fl/fl</sup>;LckCre* mice and the *Cre* negative controls was prepared for quantitative RT-PCR analysis of *Id3* transcripts. Consistent with the deletion in the *Id3* gene from the genomic sequence, the expression level of *Id3* mRNA in *LckCre* transgenic background was found to be decreased to less than 10% of control littermates (Fig. 1D).

### 3.2. Deletion of *Id3* in developing T cells perturbs T cell selection and maturation

*Id3* has been reported to play an important role during T cell selection and maturation [10]. More recently, we have reported that *Id3* deficiency promotes  $\gamma\delta$ T cell development in the C57Bl/6 background [9]. Analysis of thymocytes collected from *Id3<sup>fl/fl</sup>;LckCre* mice showed that CD4 SP cells and CD8 SP cells were decreased both in total cell numbers and relative proportion to the DP cells (Fig. 2A and B). The FACS analysis also revealed a moderate increase of  $\gamma\delta$  T cells and a decrease of mature  $\alpha\beta$  T cells in the thymus (Fig. 2C and D). DP cells undergoing positive selection are accompanied by increased expression of CD69 and TCR $\beta$ . As SP cells further mature into naïve T cells, CD69 is down regulated whereas high level TCR $\beta$  expression is maintained. Thus, an accumulation of CD69 positive and TCR $\beta$  positive cells at the DP stage would indicate a delay in positive selection [10]. Analysis with CD69 and TCR $\beta$  gated population revealed a proportional increase in CD69 positive and TCR $\beta$  positive DP cells in *Id3<sup>fl/fl</sup>;LckCre* mice, suggesting a delay in T cell development from the DP to the SP stage (Fig. 2E and F). This result is consistent with the notion that *Id3* plays a T cell intrinsic role in DP to SP selection [10]. Histological analysis of thymus section showed a grossly normal structure with a clear separation of the cortex and medulla areas (Fig. 2G). CD4 and CD8 SP cells were clearly visible in the medulla of *Id3<sup>fl/fl</sup>;LckCre* mice (Fig. 2H). Therefore, *Id3* deficient SP cells are able to enter medulla where negative selection takes place.

### 3.3. *Id3<sup>fl/fl</sup>;LckCre* mice develop Sjögren's syndrome-like phenotypes

The *Id3<sup>fl/fl</sup>;LckCre* mouse model allowed us to explicitly evaluate the role of *Id3* deficient T cells in disease development with-

out any genetic or experimental perturbation of non-T cells [11]. Similar to the phenotypes observed in *Id3-/-* mice, a reduction of saliva secretion after pilocarpine stimulation was observed in both young and aged *Id3<sup>fl/fl</sup>;LckCre* mice (Fig. 3A). Salivary glands from aged mice (8–12 months old) were examined for the presence of infiltrates with H&E staining. Lymphocyte infiltrates were observed in parotid gland and submandibular gland from *Id3<sup>fl/fl</sup>;LckCre* mice at a frequency significantly higher than those of age matched controls (Fig. 3B–F). Meanwhile, no significant lymphocyte infiltration was observed in other organs such as kidney and lung among the same experimental groups (data not shown). Serum antibody analysis among a group of 8–13 month old mice (7 *Id3<sup>fl/fl</sup>* and 7 *Id3<sup>fl/fl</sup>;LckCre* mice) did not detect autoantibodies such as anti-Ro and anti-La (data not shown). These results suggested that deletion of *Id3* in T cells is sufficient to induce most but not all clinical symptoms resembling primary Sjögren's syndrome.

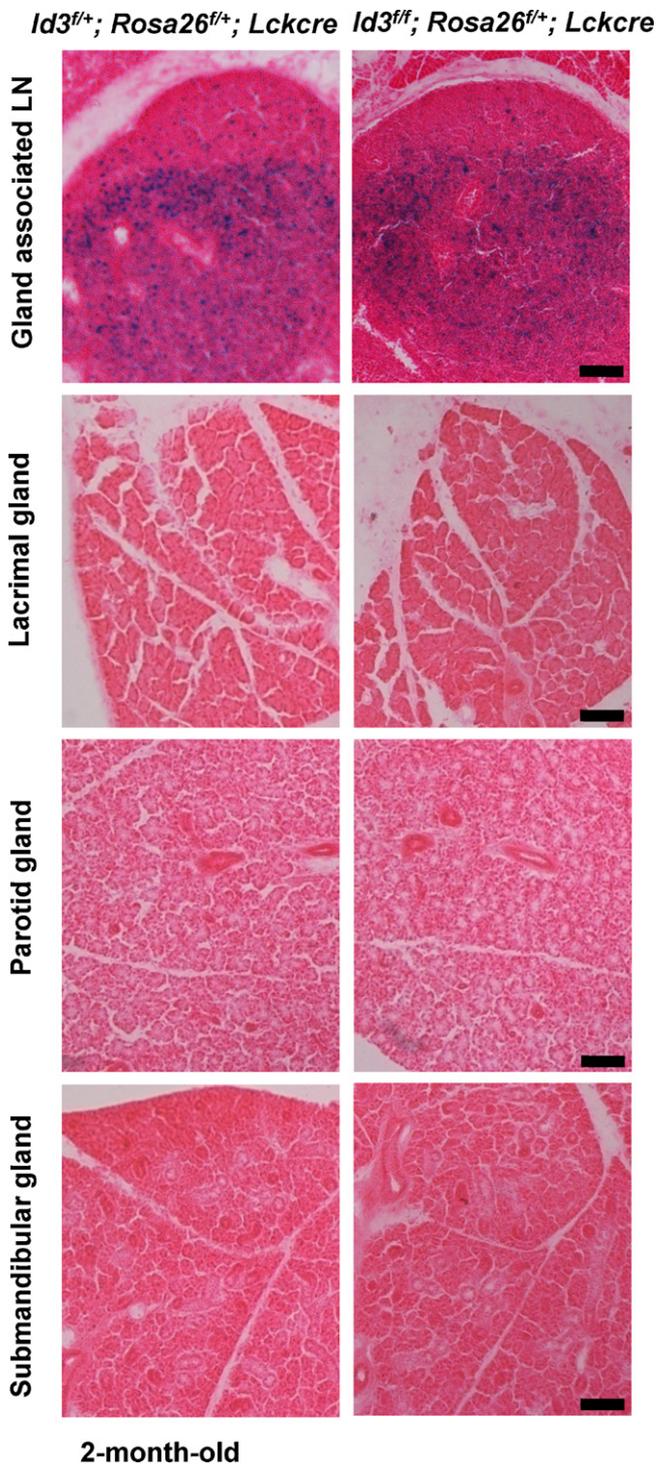
### 3.4. Absence of *Id3* deficient T cells in gland tissues

We have shown in previous studies that the impairment of gland function at the early age is not due to direct lymphocyte infiltration. However, we cannot rule out the possibility that gland function may be regulated by a small number of *Id3* deficient T cells, which are undetectable by H&E staining. To further examine the possible interaction between *Id3* deficient T cells and the gland tissues, we introduced the *Rosa26-floxstop-LacZ* allele (*Rosa26<sup>f</sup>*) as a lineage tracer to track *Id3* deficient T cells in our conditional knockout mice [21]. Because *LckCre* mediated deletion of the *Id3* gene is coupled with the activation of the LacZ marker, *Id3* deficient T cells in the periphery tissues can be detected by LacZ expression. Frozen sections of gland-associated lymph node, parotid glands, submandibular gland, and lachrymal gland from two-month old mice were stained with the LacZ substrate X-gal. While LacZ positive T cells were clearly seen in gland-associated lymph node, these labeled cells were absent in parotid glands, submandibular glands, and lachrymal glands (Fig. 4). We thus conclude that *Id3* deficient T cells may affect exocrine function without focal infiltration into the gland tissues.

### 3.5. Accumulation of activated/memory T cells occurs in aged *Id3<sup>fl/fl</sup>;LckCre* mice

Chronic autoimmune conditions are often accompanied with an accumulation of activated and/or memory T cells [22,23]. We examined T cell populations in the spleen and lymph nodes of young adult *Id3<sup>fl/fl</sup>;LckCre* mice (6-week old). The percentage and absolute number of CD4 and CD8 T cells were significantly decreased in the spleen (Fig. 5A). However, no difference was found in the cell numbers of CD4 and CD8 T cells in lymph node (data not shown). Among aged mice numbers of T cells in the spleen were similar between *Id3<sup>fl/fl</sup>;LckCre* and *Id3<sup>fl/fl</sup>* control groups (Fig. 5B). We next examined the activation/memory status of T cells using CD44 and CD62L markers. Most T cells in young adult mice are in naïve state expressing low level of CD44 ( $CD44^{low}$ ) and high level of CD62L ( $CD62L^{high}$ ). Memory T cells expressing high level of CD44 ( $CD44^{high}$ ) and low level of CD62L ( $CD62L^{low}$ ) may accumulate with age and especially under chronic stimulation of the immune system. The relative percentage of CD4 and CD8  $CD44^{high} CD62L^{low}$  memory T cells in *Id3<sup>fl/fl</sup>* controls were only slightly increased and unchanged, respectively, with age (Fig. 5C and D). In contrast, *Id3<sup>fl/fl</sup>;LckCre* mice exhibited a greater shift from naïve to memory phenotype for both CD4 and CD8 T cells from 3 to 9 months of age (Fig. 5C and D). This age dependent shift in T cell phenotype





**Fig. 4.** Absence of *Id3*-deficient T cells in gland tissues of young adult *Id3<sup>fl/fl</sup>;LckCre* mice. *Id3* deficient T cells were traced with a Cre reporter in 2 months old *Id3<sup>fl/fl</sup>;Rosa26<sup>fl/+</sup>;LckCre* and *Id3<sup>fl/+</sup>;Rosa26<sup>fl/+</sup>;LckCre* control mice. Tissue sections (20  $\mu$ m) of gland-associated lymph node, parotid salivary gland, submandibular gland, and lacrimal gland were stained with X-gal. The slides were counterstained by eosin. The scale bar equals 100  $\mu$ m.

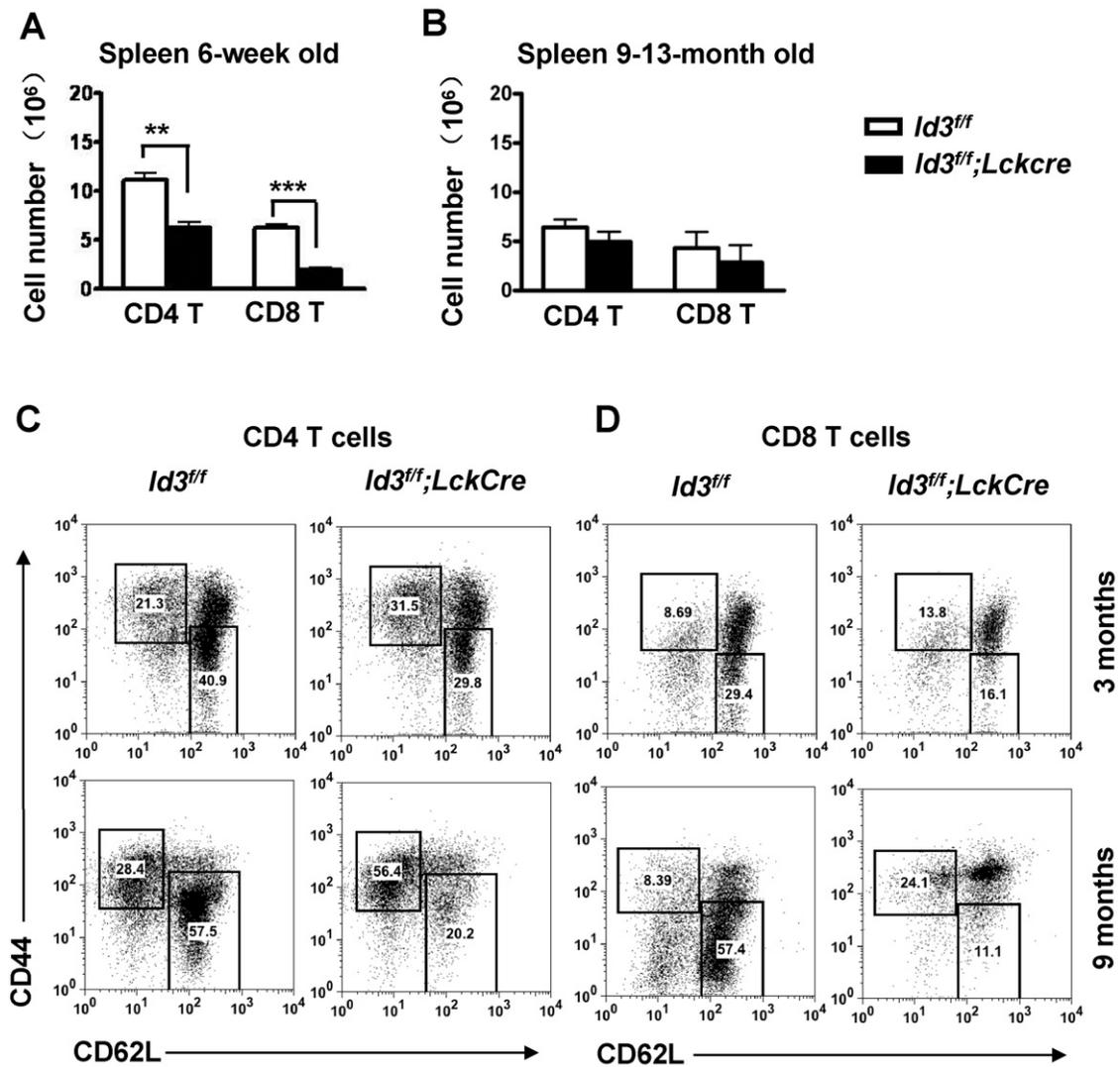
during negative selection. Another way *Id3* may perturb negative selection is through regulation of chemokine receptor expression and therefore thymocyte migration from cortex to medulla where negative selection takes place. Indeed, the level of CCR7 expression was found prematurely enhanced in DP thymocytes after removal of E-proteins in developing thymocytes [25] and moderately reduced in SP thymocytes from *Id3* deficient mice (data not shown). CCR7 deficient mice have been shown to develop autoimmune symptoms similar to Sjögren's syndrome [29]. However, thymic architecture of *Id3<sup>fl/fl</sup>;LckCre* mice was found grossly normal, implying that the moderate change in CCR7 expression is not sufficient to block the migration of most SP cells from cortex to medulla. At present, our study is not sufficient to rule out the possibility of inappropriate migration involving a small subset of thymocytes.

It is possible that *Id3* also plays important roles in T cell homeostasis, activation, effector function, or development and function of T regulatory (Treg) cells. We found *Id3<sup>fl/fl</sup>;LckCre* mice exhibit a reduced number of T cells in the spleen. However, no significant phenotypic change can be detected for these *Id3* mutant T cells at the young age when the mice begin to show impaired secretory function. This result is reminiscent of the study of *Id3* germline knockout mice. Our study also revealed a gradual change in T cell phenotypes as *Id3<sup>fl/fl</sup>;LckCre* mice became older. T cell mediated chronic conditions may result in an elevation of inflammatory cytokines. However, an analysis of serum cytokine profile from a group of 8–12 month old mice (10 *Id3<sup>fl/fl</sup>* and 11 *Id3<sup>fl/fl</sup>;LckCre* mice) failed to detect any obvious change in the level of interferon  $\gamma$ , IL2, and TNF  $\alpha$  (data not shown). Finally, we found normal number of Treg cells present in the aged *Id3<sup>fl/fl</sup>;LckCre* mice, indicating that *Id3* is not absolutely essential in the development of Treg cells (Supplemental Fig. 2). Our studies thus far are not sufficient to rule out the possibility that *Id3* is involved in the effector function of conventional and/or regulatory T cells. The disease mechanism would require additional analysis of function of *Id3* deficient T cells and other relevant effector cells. However, functional study of *Id3* deficient T cells must consider the fact that developmental defect in the thymus may impact T function later in life. The conditional knockout model with the *LckCre* transgene cannot separate the role of *Id3* in early development vs. later function in mature T cells. Therefore, the exact role of *Id3* in mature T cells would require further investigation with additional Cre system, which allows for the removal of *Id3* specifically in the mature T cells.

Although the phenotypic analysis of *Id3* conditional knockout revealed many developmental and disease features similar to that of *Id3* germline knockout, a discrepancy has also been noticed. An assessment of autoantibodies with 8–13 month old mice did not detect any anti-Ro or anti-La antibodies. In contrast, anti-Ro and anti-La antibodies have been detected in 67% *Id3* germline knockout mice at age 12 months or older [11]. Because the appearance of autoantibodies is associated with the older age of *Id3* knockout mice, we do not know whether T lineage specific *Id3* conditional knockout mice do not produce or are slow to produce autoantibodies. The role of *Id3* in humoral immunity may be further investigated after removal of *Id3* specifically in B cells or simultaneously in both T and B cells using the conditional allele.

In conclusion, our results demonstrated a T cell intrinsic role for *Id3* in regulating thymocyte development and maintaining tolerance. Our study also suggested that *Id3* deficient T cells could modulate gland secretory function without a massive infiltration into gland tissues. The experimental system established in our study provides a valuable mouse model for further investigating the molecular and cellular mechanism of primary Sjögren's syndrome.

tion against the male specific antigen *HY* is altered in *HY* specific TCR transgenic mice on the *Id3* deficient background [10]. Although the exact mechanism for *Id3* function in negative selection is not known, *Id3* has been implicated to play a role in the activation of several caspase genes in other cell types [26–28]. It remains to be determined whether *Id3* plays any role in regulating apoptosis



**Fig. 5.** Accumulation of activated/memory T cells in aged *Id3<sup>fl/fl</sup>;LckCre* mice. (A) Numbers of CD4 and CD8 T cells in the spleen from 6-week old *Id3<sup>fl/fl</sup>;LckCre* ( $n=3$ ) and *Id3<sup>fl/fl</sup>* mice ( $n=4$ ). The graphed results were means with SEM. The significance was analyzed by two-tail unpaired student's  $t$  test.  $**P=0.002$ .  $***P=0.0003$ . (B) Numbers of CD4 and CD8 T cells in the spleen of 9–13 month old *Id3<sup>fl/fl</sup>;LckCre* ( $n=4$ ) and *Id3<sup>fl/fl</sup>* mice ( $n=3$ ). Analysis was performed as in (A). No significant difference was observed between the genotype groups. (C and D) FACS plots of activated/memory T cells (CD44<sup>high</sup>CD62L<sup>low</sup>, the upper left rectangle gate) and naïve T cells (CD44<sup>low</sup>CD62L<sup>high</sup>, the lower right rectangle gate) among CD4 T cells (C) and CD8 T cells (D). Gated CD4 or CD8 lymphocytes are from spleen of 3-month old (upper panel of C and D) and 9-month old mice (bottom panel of C and D). Results are representative of at least three mice for each age and genotype group.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2010.09.009.

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