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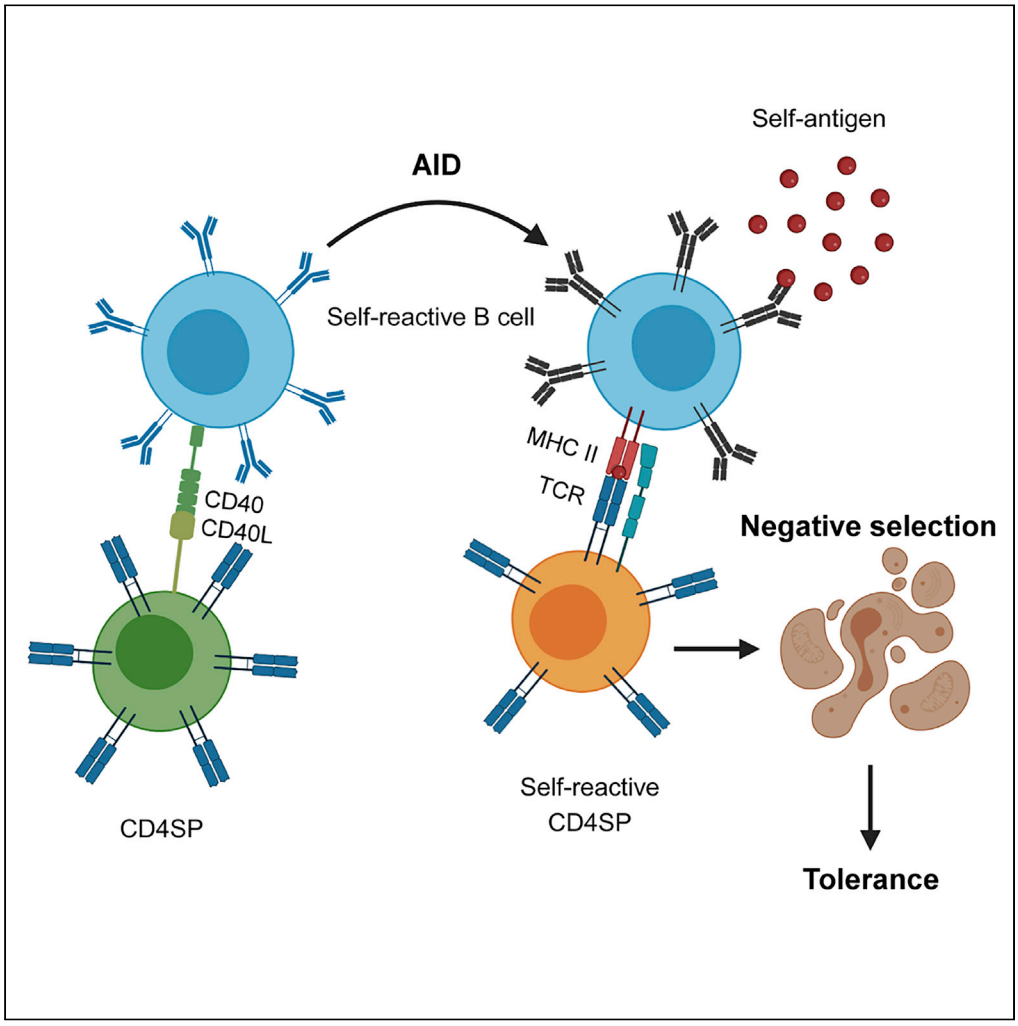


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## Highlights

Thymic B cells promote the negative selection of CD4SP thymocytes in polyclonal settings

AID deficiency impairs the elimination of pathogenic self-reactive thymocytes

AID expression by thymic B cells reduces autoimmune susceptibility

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# Activation-induced cytidine deaminase expression by thymic B cells promotes T-cell tolerance and limits autoimmunity

Félix Lombard-Vadnais,[1,2] Geneviève Chabot-Roy,[1] Astrid Zahn,[3] Sahily Rodriguez Torres,[1,4] Javier M. Di Noia,[2,3,5,6] Heather J. Melichar,[1,5,*] and Sylvie Lesage[1,4,7,*]

## SUMMARY

**Elimination of self-reactive T cells in the thymus is critical to establish T-cell tolerance. A growing body of evidence suggests a role for thymic B cells in the elimination of self-reactive thymocytes. To specifically address the role of thymic B cells in central tolerance, we investigated the phenotype of thymic B cells in various mouse strains, including non-obese diabetic (NOD) mice, a model of autoimmune diabetes. We noted that isotype switching of NOD thymic B cells is reduced as compared to other, autoimmune-resistant, mouse strains. To determine the impact of B cell isotype switching on thymocyte selection and tolerance, we generated NOD.AID$^{-/-}$ mice. Diabetes incidence was enhanced in these mice. Moreover, we observed reduced clonal deletion and a resulting increase in self-reactive CD4$^{+}$ T cells in NOD.AID$^{-/-}$ mice relative to NOD controls. Together, this study reveals that AID expression in thymic B cells contributes to T-cell tolerance.**

## INTRODUCTION

Developing T cells undergo a series of selection processes in the thymus to yield a functional repertoire of T cells able to recognize antigens in the context of major histocompatibility complex (MHC) molecules, while ensuring tolerance to self-antigens.[1–3] Selection of thymocytes is mediated by various antigen-presenting cells (APCs), such as thymic epithelial cells (TECs), conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs).[2] In addition, human and mouse thymi also harbor a small population of B cells.[4,5] In mice, B cells represent ~0.1-0.3% of total thymic cells and are mostly found at the cortico-medullary junction.[6–8] Although B cells are not considered efficient phagocytes, they take advantage of their antigen receptor (B cell receptor, BCR) to efficiently capture antigens from the environment and present peptides from these proteins via MHC II molecules.[9,10] Thymic B cells express high levels of MHC II as well as co-stimulatory molecules and contribute to thymocyte education in some settings.[6] Negative selection is the process by which thymocytes bearing T-cell receptors (TCR) with high affinity to self-peptide MHC complexes are purged via apoptosis.[11,12] Notably, in antigen receptor transgenic mice with monoclonal T and B cell repertoires, thymic B cells can promote the elimination of self-reactive CD4 single positive (SP) thymocytes.[6,7,13] Still, the factors contributing to thymic B cell-mediated negative selection are incompletely understood.

A recent study suggests that thymic B cells that have undergone class switch recombination (CSR) to change BCR isotype may promote central T-cell tolerance.[14] It was shown that CSR can occur directly in the thymus and is dependent on thymocyte-derived signals.[14] IgG$^{+}$ and IgA$^{+}$ thymic B cells were shown to express activation-induced cytidine deaminase (AID). Importantly, the specificity of the BCR repertoire of isotype-switched thymic B cells appears to be skewed toward self-antigens.[14] This finding led to the hypothesis that self-reactive isotype-switched thymic B cells may facilitate the negative selection of self-reactive thymocytes. Yet, whether isotype-switched thymic B cells directly induce the negative selection of autoreactive thymocytes has not been assessed. More importantly, due to the redundancy in both central and peripheral tolerance processes for preventing autoimmune diseases, it is not clear whether the elimination of self-reactive thymocytes by isotype-switched thymic B cells has a functional impact on the development of autoimmunity.

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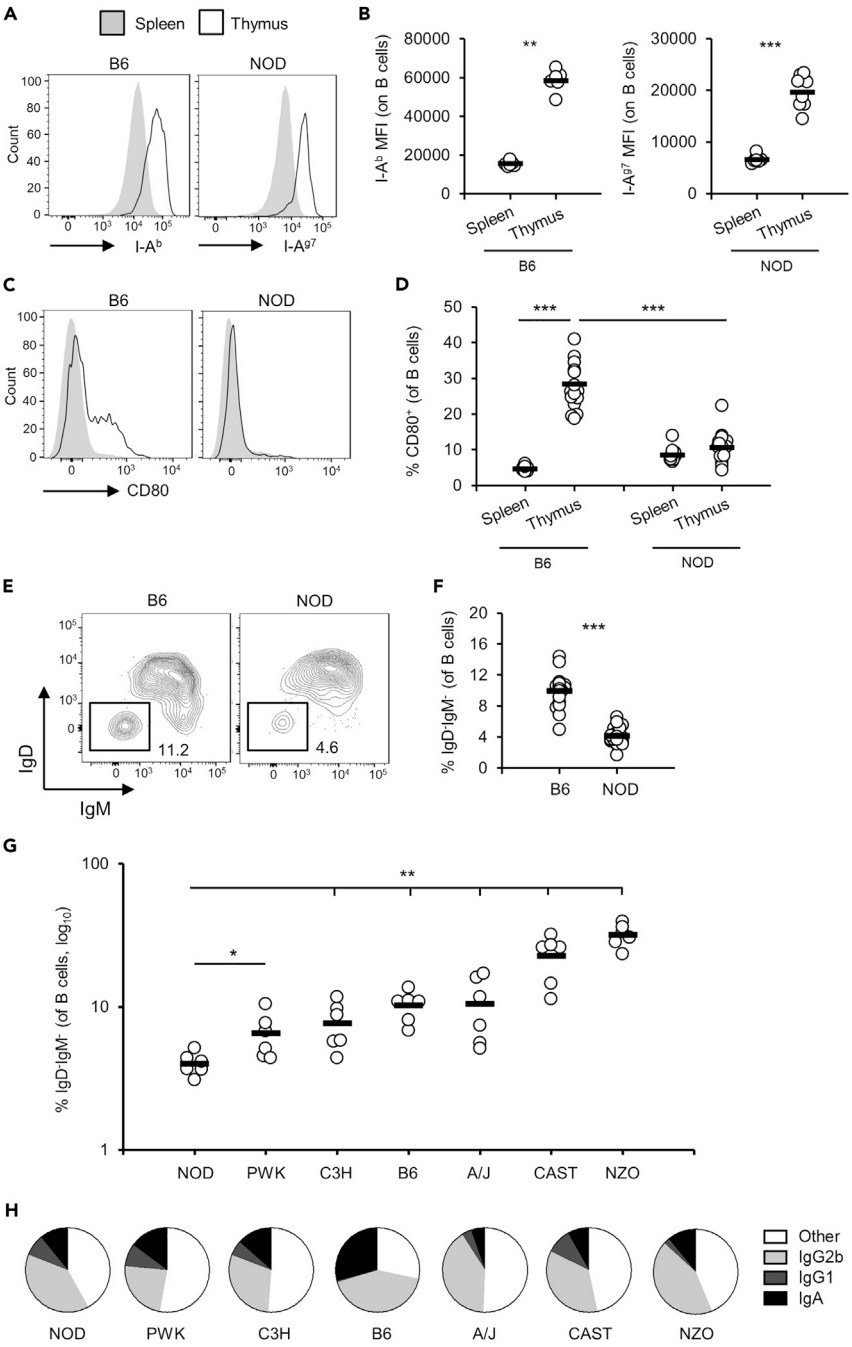


**Figure 1. Unique characteristics of thymic B cells from NOD mice**

(A) Representative flow cytometry profiles and (B) quantification of MHC II expression on B cells from the spleen and the thymus of B6 and NOD mice (n = 7-8, Mann-Whitney U test), measured by mean fluorescence intensity (MFI).

(C) Representative flow cytometry profiles of CD80 expression on splenic and thymic B cells ($CD19^+B220^+$) from B6 and NOD mice.

(D) Compilation of the percentage of $CD80^+$ B cells from the spleen and thymus of B6 and NOD mice (n > 14, Mann-Whitney U test).

(E) Representative flow cytometry profiles of IgM and IgD expression on thymic B cells from B6 and NOD mice. Scaling = 5% with outliers shown for contour plots.

**Figure 1. *Continued***

(F) Compilation of the percentage of $IgM^{-}IgD^{-}$ thymic B cells from B6 and NOD mice (n > 16, Mann-Whitney U test).

(G) Compilation of the percentage of $IgM^{-}IgD^{-}$ thymic B cells from genetically divergent mouse strains (n = 6, one-way ANOVA).

(H) Distribution of antibody subclasses expressed by $IgM^{-}IgD^{-}$ thymic B cells from the same mice as in (G). "Other" refers to B cells with a $IgM^{-}IgD^{-}IgA^{-}IgG1^{-}IgG2b^{-}$ phenotype.

The data was acquired in at least three independent experiments. Dots represent data from individual mice and the dash depicts the mean. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. See also Figure S1.

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin-producing pancreatic β cells.[15] The non-obese diabetic (NOD) mouse strain is autoimmune-prone[16] and spontaneously develops autoimmune diabetes.[17–19] T cells are necessary and sufficient for autoimmune diabetes onset and progression,[20–22] and it has been suggested that defects in central T-cell tolerance in NOD mice contribute to the development of autoimmune diabetes.[23–28] Intriguingly, the defects in central tolerance correlate with abnormal B cell development in the thymus of NOD mice.[29] Still, the contribution of thymic B cells to T-cell tolerance in NOD mice is unknown.

To investigate the potential contributions of thymic B cells to T-cell tolerance and autoimmunity, we phenotyped B cells from the thymus of various mouse strains, including NOD mice. As compared to genetically divergent mouse strains, we observe that NOD thymic B cell CSR is inefficient. Interestingly, this trait correlates with reduced tolerance of thymocytes toward self-antigens. We also show that the disruption of AID, which prevents CSR, impairs the negative selection of CD4SP thymocytes and favors the development of autoimmunity. Our data reveal a new role for AID in the thymus in T-cell tolerance, whereby thymic B cell expression of AID promotes the negative selection of self-reactive thymocytes and has a functional impact on autoimmune disease susceptibility.

## RESULTS

### Reduction of co-stimulation and class switch recombination in thymic B cells from non-obese diabetic mice

To investigate the potential role of thymic B cells in impaired T-cell tolerance in NOD mice, we analyzed the phenotype of thymic B cells from 7- to 10-week-old non-diabetic B6 and NOD mice (Figure S1A). As compared to splenic B cells, thymic B cells from both strains expressed higher levels of MHC II molecules (Figures 1A and 1B). In contrast, while a higher percentage of thymic B cells from B6 mice express CD80 relative to B cells isolated from the spleen (Figures 1C and 1D),[6] thymic B cells from NOD mice expressed lower levels of this co-stimulatory molecule as compared to their B6 counterparts (Figures 1C and 1D). This low level of CD80 expression in the NOD thymus is limited to thymic B cells, as other thymic APCs express levels of CD80 that are comparable to those observed in B6 mice (Figures S1B and S1C). This reveals that thymic B cells in NOD mice have a distinct phenotype relative to other thymic APCs.

CD80 expression on APCs is typically induced following activation.[30,31] The low expression of this co-stimulatory molecule on thymic B cells in NOD mice suggest that they may not be effectively activated. This is not due to defects in their response to stimuli; thymic B cells from NOD mice efficiently upregulate the expression of co-stimulatory molecules and activation markers following *in vitro* activation with LPS or αCD40 antibodies (Figure S1D). Therefore, the reduced expression of CD80 on thymic B cells from NOD mice relative to B6 mice suggests that the NOD thymic environment provides less B cell activation signals *in vivo*. Activation of thymic B cells can also lead to the expression of AID and, consequently, to CSR.[14] In line with the low level of CD80 expression, a lower percentage of $IgM^{-}IgD^{-}$ isotype-switched thymic B cells was observed in NOD mice relative to B6 mice (Figures 1E and 1F). To determine if the relatively higher level of thymic B cell CSR is a specific feature of the B6 strain,[14] we analyzed thymic B cells from multiple genetically divergent mouse strains: the common C3H, A/J and NZO strains as well as the more recently wild-derived PWK and CAST inbred strains (Figure S1E). Isotype-switched $IgM^{-}IgD^{-}$ thymic B cells were observed in the thymus of all tested strains (Figure 1G), with most of those cells expressing an IgG or IgA BCR (Figure 1H). Among these genetically divergent mouse strains, the NOD mouse, the only strain spontaneously developing autoimmunity, displays the lowest percentage of isotype-switched thymic B cells (Figure 1G). Altogether, these data suggest that thymic B cells in NOD mice express lower levels of co-stimulatory molecules and exhibit less CSR than the other mouse strains tested.

## The thymic B cell traits from non-obese diabetic mice correlate with reduced T-cell tolerance to self-antigens

It has been recently suggested that isotype-switched thymic B cells may promote central T-cell tolerance.[14] Using the genetically divergent mouse strains, we determined that the percentage of isotype-switched thymic B cells varies tremendously (Figure 1G). We took advantage of this variability to assess whether the percentage of isotype-switched thymic B cells correlated with thymic phenotypes. Interestingly, we found that the percentage of $IgM^{-}IgD^{-}$ thymic B cells correlated with the percentage of $CD69^{+}$ CD4SP thymocytes (Figure 2A). Notably, CD69 expression is induced following TCR stimulation, during positive and negative selection.[32] In contrast to CD4SP thymocytes, no correlation between isotype-switched B cells and CD69 upregulation was observed for CD8SP thymocytes (Figure 2B). These results suggest that isotype-switched thymic B cells, which express high levels of MHC II, may participate in the thymic selection of CD4SP thymocytes.

We observed a greater percentage and number of CD4SP, but not of CD8SP, thymocytes in NOD mice as compared to B6 mice (Figures 2C and S1F), suggesting a potential defect in the negative selection of CD4SP thymocytes in NOD mice. In addition, among these CD4SP thymocytes, the percentage of active caspase-$3^{+}$ cells, a marker of cells undergoing clonal deletion,[33] was reduced in NOD mice (Figure 2D). These results suggest that the negative selection of CD4SP thymocytes is less effective in NOD mice. As a result, CD4SP thymocytes from NOD mice should exhibit enhanced self-reactivity. To assess the level of self-reactivity of CD4SP thymocytes from B6 and NOD mice, we performed an *in vitro* proliferation assay of CD4SP thymocytes on activated splenic B cells (Figure 2E). Specifically, splenic B cells from both strains were activated using αCD40 antibodies and used as a source of APCs and self-antigens. *In vitro* activation induced high levels of CD80 and CD86 on B cells from both strains (Figure S1G). CFSE-labeled CD4SP thymocytes were co-cultured for three days with activated B cells from the respective strains. At the end of the co-culture, CD4SP thymocyte proliferation and activation were quantified by flow cytometry. While the proliferation of CD4SP thymocytes in the B6 co-culture was minimal, a greater proportion of CD4SP thymocytes from NOD mice proliferated (Figures 2F and 2G). This increase in $CFSE^{lo}$ cells was not caused by the more rapid proliferation of NOD thymocytes, as CD4SP cells from B6 and NOD mice had a similar proliferation index (Figure 2H). Altogether, these results demonstrate that the low percentage of CSR in thymic B cells in NOD mice correlates with a decrease in the induction of negative selection at the CD4SP stage and a corresponding increase in self-reactive CD4SP thymocytes in NOD relative to B6 mice.

## Loss of activation-induced cytidine deaminase in thymic B cells promotes the development of autoimmune diabetes

To study the impact of AID expression in thymic B cells on the development of autoimmunity, we generated a $NOD.AID^{-/-}$ strain. As expected, $NOD.AID^{-/-}$ mice completely lack CSR in thymic B cells (Figure 3A). Genetic deletion of AID does not impact thymic B cell expression of MHC II, CD80, or CD69 (Figure S2A). There was also no significant difference in the abundance of B cells in the thymus of $NOD.AID^{+/+}$ and $NOD.AID^{-/-}$ mice (Figure S2B). First, to test the impact of B cell CSR on the development of autoimmunity, we performed a diabetes incidence study. Compared to $NOD.AID^{+/+}$ littermates, diabetes onset was accelerated and diabetes incidence was higher in $NOD.AID^{-/-}$ mice (Figure 3B). In addition to the increased diabetes incidence and similar to previous reports,[34] $NOD.AID^{-/-}$ mice exhibited an enlargement of the spleen and pancreatic lymph nodes (Figures 3C and 3D), where diabetogenic T cells are primed by self-antigens.[35,36] In contrast, skin-draining lymph nodes were not enlarged in $NOD.AID^{-/-}$ mice relative to littermate controls (Figures 3D and S2C). In the spleen and pancreatic lymph nodes, we also observed an increase in the percentage and number of activated $CD4^{+}$ T cells, as detected by both the increase in expression of CD69 and an increase in $CD62L^{lo}CD44^{hi}$ $CD4^{+}$ T cells (Figures 3E, 3F, S2D, and S2E). No increase in activated T cells was observed in skin-draining lymph nodes (Figures 3E, 3F, and S2E). The T-cell activation phenotype is limited to $CD4^{+}$ T cells, as no difference in the percentage of activated $CD8^{+}$ T cells was observed between $NOD.AID^{+/+}$ and $NOD.AID^{-/-}$ mice in any of the organs tested (Figure 3G). Together, these data suggest that a lack of AID expression increases diabetes incidence in NOD mice and is associated with an increase in activated $CD4^{+}$ T cells, but not $CD8^{+}$ T cells, in the spleen and pancreatic lymph nodes, where priming of islet antigen-specific T cells takes place.[35,36]

AID expression impacts multiple aspects of B cell biology including antibody production and germinal center formation.[37,38] It is, therefore, possible that the increased diabetes onset in $AID^{-/-}$ NOD mice (Figure 3B) is a consequence of altered B cell function in the periphery rather than an impact on the

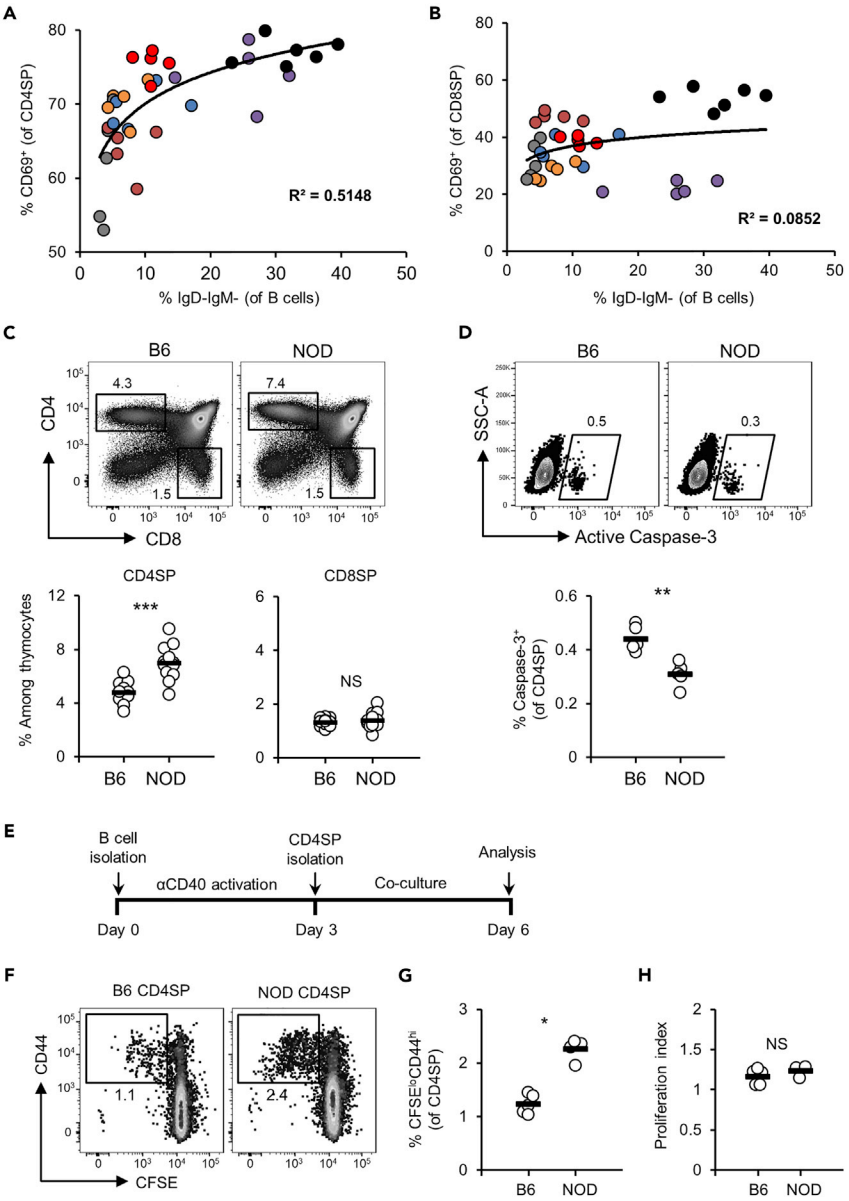


**Figure 2. NOD thymic B cell traits correlate with reduced T-cell tolerance to self-antigens**

Correlation between the percentage of isotype-switched thymic B cells and the expression of CD69 on (A) CD4SP and (B) CD8SP thymocytes from the same mice as in Figure 1G. Each color represents a different mouse strain.

(C) Representative flow cytometry profiles (top panel) and compilation (bottom panel) of CD4SP and CD8SP subsets in the thymus of B6 and NOD mice (n = 10-11, Mann-Whitney U test).

(D) Representative flow cytometry profiles (top panel) and percentage (bottom panel) of active caspase-3$^{+}$ CD4SP thymocytes from B6 and NOD mice (n = 5, Mann-Whitney U test).

(E) Schematic of the *in vitro* co-culture assay used in F-H.

(F) Representative flow cytometry profiles of CFSE and CD44 expression on CD4SP thymocytes from the co-cultures.

(G) Compilation of the percentage of CFSE$^{lo}$CD44$^{hi}$ cells in thymocytes from the co-cultures (n = 4-5, Mann-Whitney U test).

(H) Proliferation index of the thymocytes at the end of the co-cultures (n = 4-5, Mann-Whitney U test).

The data was acquired in at least two independent experiments. Dots represent data from individual mice and the dash denotes the mean. NS, non-significant $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. See also Figure S1.

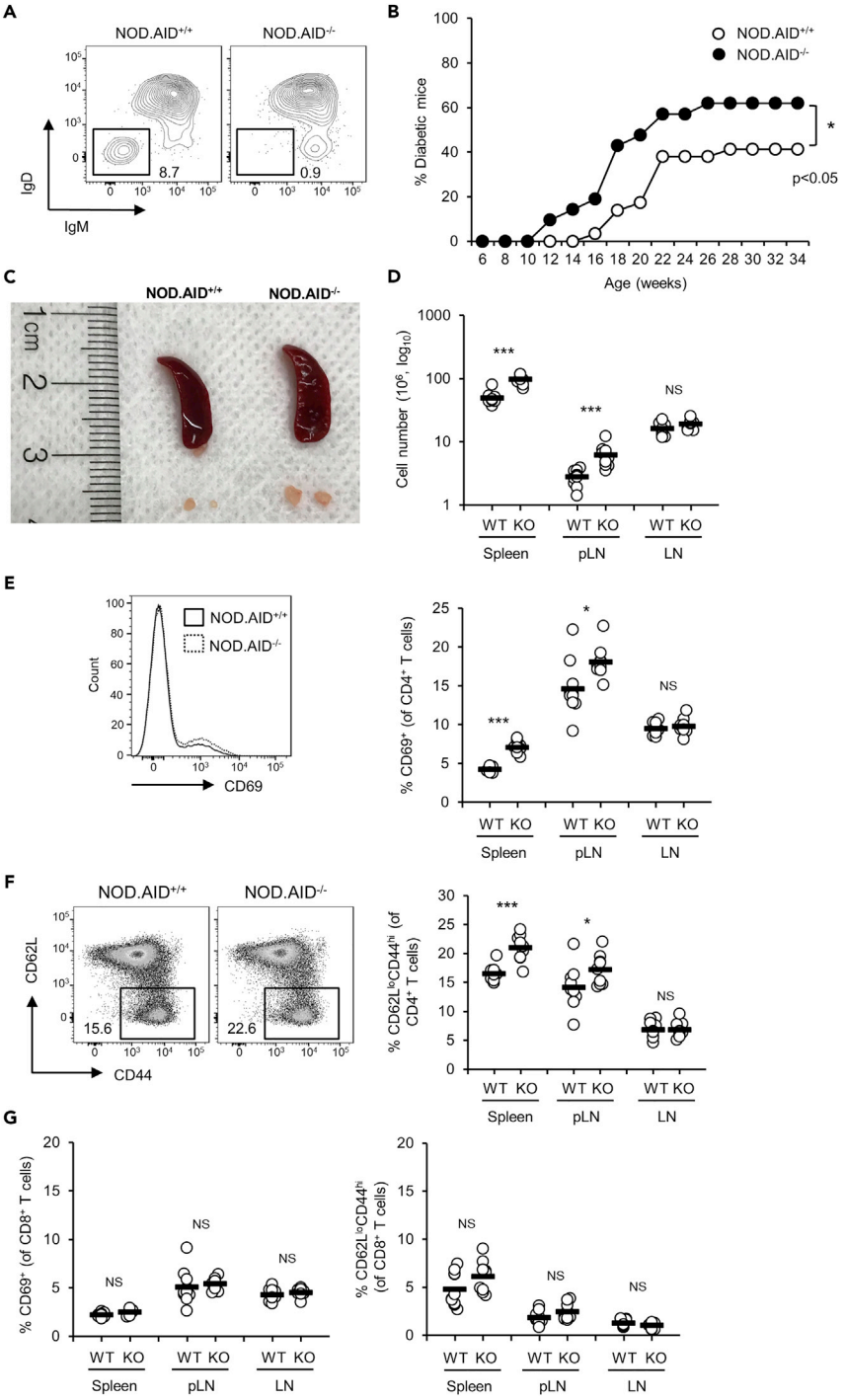


**Figure 3. AID deficiency promotes the development of autoimmune diabetes**

(A) Representative flow cytometry profiles of IgM and IgD expression on thymic B cells from NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice. Scaling = 5% with outliers shown for contour plots.

(B) Diabetes incidence of female NOD.AID$^{+/+}$ (n = 29) and NOD.AID$^{-/-}$ (n = 21) mice (Log Rank (Mantel-Cox) test).

(C) Image of representative spleens and pancreatic lymph nodes from non-diabetic NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice.

(D) Absolute numbers of cells in the spleen, pancreatic and skin-draining lymph nodes from non-diabetic NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice (n = 9-10, Mann-Whitney U test).

**Figure 3. *Continued***

(E) Representative flow cytometry profiles of CD69 expression on splenic $CD4^+$ T cells (left panel) and compilation of the percentage (right panel) of $CD69^+$ $CD4^+$ T cells from the spleen, pancreatic lymph nodes, and skin-draining lymph nodes of non-diabetic NOD.$AID^{+/+}$ and NOD.$AID^{-/-}$ mice (n = 9-10, Mann-Whitney U test).

(F) Representative flow cytometry profiles of CD62L and CD44 expression on splenic $CD4^+$ T cells (left panel) and compilation of the percentage (right panel) of $CD62L^{lo}CD44^{hi}$ $CD4^+$ T cells from the spleen, pancreatic lymph nodes, and skin-draining lymph nodes of non-diabetic NOD.$AID^{+/+}$ and NOD.$AID^{-/-}$ mice (n = 9-10, Mann-Whitney U test).

(G) Compilation of the percentage of $CD69^+$ (left panel) and $CD62L^{lo}CD44^{hi}$ (right panel) $CD8^+$ T cells from the spleen, pancreatic lymph nodes, and skin-draining lymph nodes of non-diabetic NOD.$AID^{+/+}$ and NOD.$AID^{-/-}$ mice (n = 9-10, Mann-Whitney U test).

The data was acquired in at least three independent experiments. Dots represent data for individual mice and the dash denotes the mean. NS, non-significant $p > 0.05$; *, $p < 0.05$; ***, $p < 0.001$. See also Figure S2.

diabetogenic potential of T cells. To test this possibility, we used lymphopenic NOD.$Rag^{-/-}$ mice, which lack all T and B lymphocytes and therefore do not progress to diabetes.[39] Adoptive transfer of T cells into NOD.$Rag^{-/-}$ mice induces autoimmune diabetes.[39] When compared to recipients of total peripheral T cells from NOD.$AID^{+/+}$ mice, recipients of T cells from NOD.$AID^{-/-}$ mice showed accelerated diabetes onset (Figure 4A), indicating that a lack of AID impacts T-cell diabetogenicity. This result also suggests that the increased diabetes onset is observed in NOD.$AID^{-/-}$ mice (Figure 3B) is, at least in part, T cell-mediated.

Peripheral T-cell tolerance mechanisms potentially contribute to the diabetogenic potential of T cells in NOD.$AID^{-/-}$ mice. To determine if loss of AID in thymic B cells alters the diabetogenic potential of thymocytes, we transferred total thymocytes from NOD.$AID^{+/+}$ and NOD.$AID^{-/-}$ mice into NOD.$Rag^{-/-}$ recipients. We collected pancreas 10 weeks post-transfer and quantified insulitis (Figure 4B). Recipients of thymocytes from NOD.$AID^{-/-}$ mice showed comparably more insulitis than the recipients of thymocytes from NOD.$AID^{+/+}$ mice (Figure 4C). Consistent with the increase of activated $CD4^+$ T cells in NOD.$AID^{-/-}$ mice (Figures 3E and 3F), an increase of activated $CD4^+$ T cells was observed in the spleen and pancreatic lymph nodes of mice receiving thymocytes from NOD.$AID^{-/-}$ mice (Figure 4D). Once again, the activation of $CD8^+$ T cells was not affected (Figure 4E). Altogether, our results suggest that CD4SP self-reactive thymocytes are not effectively eliminated in mice lacking AID expression and that AID-expressing thymic B cells likely mediate the negative selection of CD4SP self-reactive thymocytes. Moreover, these self-reactive thymocytes can cause significant lesions in the pancreas.

## Activation-induced cytidine deaminase deletion impairs the negative selection of CD4SP thymocytes

We next aimed to determine whether the negative selection of thymocytes was impaired in the absence of AID. As a measure of negative selection, we quantified the percentage of thymocytes from NOD.$AID^{+/+}$ and NOD.$AID^{-/-}$ mice expressing active caspase-3 (Figure S3A). A lower percentage of CD4SP thymocytes from NOD.$AID^{-/-}$ mice expressed active caspase-3 than NOD.$AID^{+/+}$ littermates (Figures 5A and 5B). In contrast, no significant difference was observed in double-positive (DP) and CD8SP thymocytes (Figures 5A and 5B). In addition to active caspase-3, we quantified HELIOS expression. HELIOS is induced in self-reactive CD4SP thymocytes following strong TCR signals and can be used as a marker of negative selection.[40] Notably, as $CD4^+FOXP3^+$ regulatory T cells (Tregs) also express HELIOS, Tregs were excluded from the analysis (Figure 5C). As for active caspase-3, we found a significant reduction of $HELIOS^+FOXP3^-$ CD4SP thymocytes in NOD.$AID^{-/-}$ mice relative to NOD.$AID^{+/+}$ littermates (Figures 5C and 5D). These results are consistent with impaired negative selection of self-reactive CD4SP thymocytes in NOD.$AID^{-/-}$ mice.

The decrease in negative selection of thymocytes in NOD.$AID^{-/-}$ mice could be due to a reduction of interactions with thymic B cells or by an impaired capacity of thymic B cells to induce TCR signaling. To test if interactions between thymic B cells and thymocytes are affected by AID deletion, we quantified interactions using flow cytometry. Co-expression of B220 and CD19 was used to identify thymic B cells interacting with DP, CD4SP, and CD8SP thymocyte subsets (Figures 5E and S3B). Expectedly, doublets were larger in size, as defined by the FSC/SSC profile (Figure S3B). Thymic B cells preferentially interacted with CD4SP thymocytes (Figures 5E and 5F). Interestingly, a significant reduction of doublets was observed between thymic B cells and CD4SP thymocytes from NOD.$AID^{-/-}$ mice as compared to cells from NOD.$AID^{+/+}$

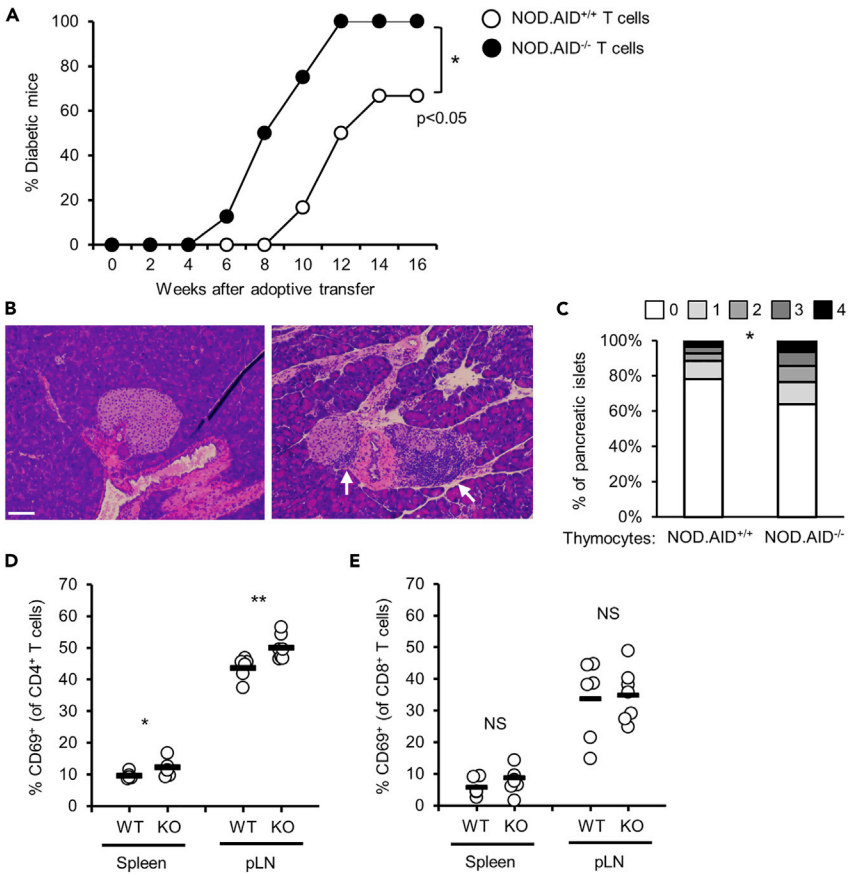


**Figure 4. AID deletion impacts T-cell diabetogenicity and enhances insulitis**

(A) Diabetes incidence of female NOD.Rag$^{-/-}$ mice that received 6-8 x $10^6$ total splenic T cells from age-matched non-diabetic female NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice (n = 6-8, Log Rank [Mantel-Cox] test).

(B) Representative images of an intact (left) and infiltrated (right) islet from the pancreas of a NOD.Rag$^{-/-}$ mouse 10 weeks after intravenous injection of 22 x $10^6$ total NOD.AID$^{-/-}$ thymocytes (scale bar represents 25 μm).

(C) Insulitis score for female NOD.Rag$^{-/-}$ mice 10 weeks post-injection of 22 x $10^6$ total thymocytes from adult female NOD.AID$^{+/+}$ or NOD.AID$^{-/-}$ mice (n = 6-7, Chi-square test). Scale: 0 = no infiltration, 1 = peri-insulitis, 2 = infiltration <50%, 3 = infiltration >50%, 4 = complete infiltration.

Compilation of the percentage of CD69$^+$ CD4$^+$ (D) and CD8$^+$ (E) T cells at 10 weeks post-injection of thymocytes for the mice described in (C) (n = 6-7, Mann-Whitney U test).

The data was acquired in at least three independent experiments. NS, non-significant p > 0.05; *, p < 0.05; **, p < 0.01. Dots represent data from individual mice and the dash denotes the mean.

mice (Figure 5F), suggesting that AID expression by thymic B cells promotes interactions with CD4SP thymocytes.

In the absence of AID, there are fewer interactions between thymic B cells and CD4SP thymocytes as well as fewer CD4SP thymocytes expressing negative selection markers. Thymocytes from these NOD.AID$^{-/-}$ mice also promote insulitis. This suggests that there may be an accumulation of self-reactive CD4SP thymocytes in NOD.AID$^{-/-}$ mice. To test this hypothesis, we assessed the tolerance of CD4SP thymocytes to self-antigens in NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice as before (Figure 2E). Relative to CD4SP thymocytes from NOD.AID$^{+/+}$ mice, a greater percentage of CD4SP thymocytes from NOD.AID$^{-/-}$ mice was activated and proliferated in response to activated B cells isolated from NOD mice (Figures 5G-5I). The absence of AID thus leads to the accumulation of autoreactive CD4SP thymocytes and a reduction of tolerance to self-antigens. Together, these results suggest that AID expression by thymic B cells promotes the negative selection of self-reactive thymocytes and improves T-cell tolerance.

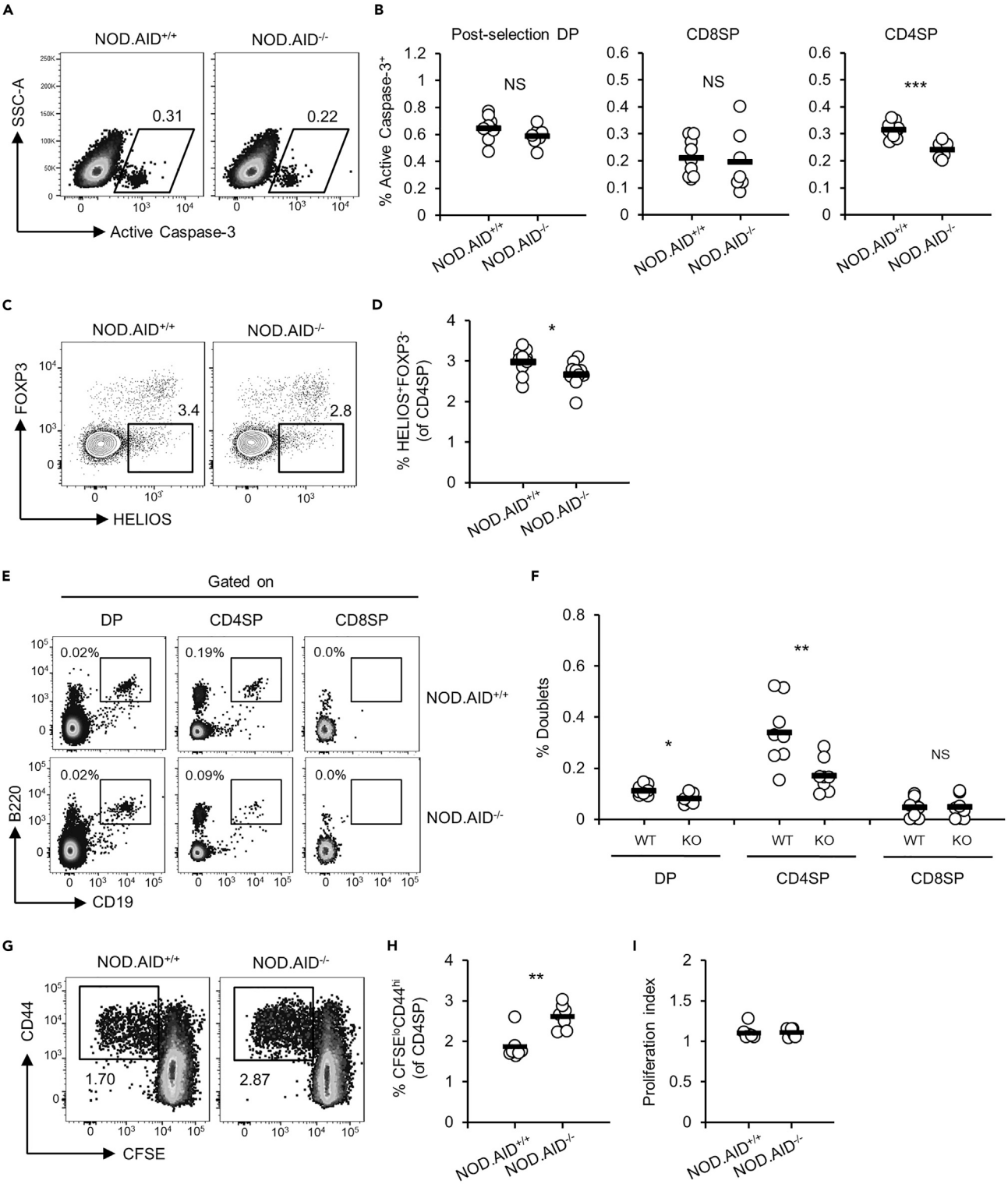


**Figure 5. AID deletion impairs the negative selection of CD4SP thymocytes**

(A) Representative flow cytometry profiles of active Caspase-3 expression in CD4SP thymocytes and (B) compilation of the percentage of active caspase-3$^{+}$ DP, CD4SP, and CD8SP thymocytes from NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice (n = 8-10, Mann-Whitney U test).

(C) Representative flow cytometry profiles and (D) compilation of the percentage of HELIOS$^{+}$ CD4SP thymocytes from NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice (n = 9-13, Mann-Whitney U test).

**Figure 5. *Continued***

(E) Representative flow cytometry profiles of *ex vivo* DP, CD4SP, and CD8SP thymocytes conjugated with thymic B cells from NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice.

(F) Compilation of the percentage of DP, CD4SP, and CD8SP thymocytes conjugated to thymic B cells from NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice normalized to the percentage of total B cells in the thymus of each mouse (n = 8-9, Mann-Whitney U test).

(G) Representative flow cytometry profiles of CFSE and CD44 expression on CD4SP thymocytes from NOD.AID$^{+/+}$ and NOD.AID$^{-/-}$ mice co-cultured with activated splenic B cells from NOD.CD45.2$^{+}$ mice.

(H) Compilation of the percentage of CFSE$^{lo}$CD44$^{hi}$ cells in thymocytes from the co-cultures (n = 7, Mann-Whitney U test).

(I) Proliferation index of the thymocytes at the end of the co-cultures (n = 7).

The data was acquired in at least three independent experiments. Dots represent data from individual mice and the dash denotes the mean. NS, non-significant $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. See also Figure S3.

## Activation-induced cytidine deaminase expression in B cells promotes the clonal deletion of self-reactive thymocytes

The efficacy of antigen-specific negative selection is difficult to quantify in a non-transgenic setting, where thymocytes express a polyclonal TCR repertoire. To determine the extent to which AID impacts the negative selection of CD4SP thymocytes in a setting distinct from NOD.AID$^{-/-}$ mice, we turned to an MHC class-II restricted TCR transgenic mouse model on the B10.BR genetic background. In 3A9 TCR transgenic mice, most thymocytes express the MHC II (I-A$^{k}$) restricted 3A9 TCR specific for a peptide from the hen egg lysozyme (HEL) protein. In the absence of HEL, thymocytes complete their differentiation into CD4SP thymocytes (Figures 6A and 6B, 3A9 TCR$^{+}$ HEL$^{-}$ AID$^{+/+}$). When the HEL transgene driven by the rat insulin promoter is also expressed, soluble HEL is detected in circulation as well as in the thymus.[24,41] In these TCR:HEL double transgenic mice, most CD4SP thymocytes are eliminated by negative selection (Figures 6A and 6B, 3A9 TCR$^{+}$ HEL$^{+}$ AID$^{+/+}$).[23] We crossed the TCR:HEL double transgenic mice onto the AID$^{-/-}$ background to quantify the clonal deletion of self-reactive CD4SP thymocytes in the absence of AID expression by thymic B cells. In the absence of HEL, AID disruption did not impact the differentiation of 3A9 TCR$^{+}$ CD4SP thymocytes (Figures 6A and 6B, 3A9 TCR$^{+}$ HEL$^{-}$ AID$^{-/-}$), suggesting that the AID deficiency does not impact the positive selection of thymocytes. In contrast, in the presence of HEL (Figures 6A and 6B, 3A9 TCR$^{+}$ HEL$^{+}$ AID$^{-/-}$), an $\sim$ 20% increase of self-reactive 3A9 TCR$^{+}$ CD4SP thymocytes was observed in the thymus of AID$^{-/-}$ mice as compared to AID$^{+/+}$ controls, indicating a significant reduction in clonal deletion (Figures 6A and 6B). In addition, interactions between thymic B cells and CD4SP thymocytes, which were very rare in the absence of the cognate antigen, were increased in mice expressing HEL (Figure 6C). Interestingly, 3A9 TCR$^{+}$ CD4SP thymocytes that were interacting with thymic B cells *in vivo* showed significantly lower expression of the 3A9 TCR than CD4SP thymocytes that were not interacting with B cells (Figure 6D). This observation supports the notion that thymic B cells can induce TCR signaling in CD4SP thymocytes in an antigen-specific manner, as TCR downregulation is a consequence of strong TCR signaling during thymocyte selection.[42] Overall, these results suggest that AID expression in polyclonal thymic B cells can have a significant impact on the negative selection of thymocytes.

## Deletion of activation-induced cytidine deaminase in B6 mice limits negative selection and induces insulitis

The impact of AID expression by thymic B cells on diabetes and insulitis was studied in NOD mice, which are prone to pancreatic autoimmunity. To test the impact of AID expression in thymic B cells in mice that do not spontaneously develop autoimmunity, we compared negative selection and insulitis in B6.AID$^{+/+}$ and B6.AID$^{-/-}$ mice. As previously observed in NOD.AID$^{-/-}$ mice (Figures 5A and 5B), CD4SP thymocytes from B6.AID$^{-/-}$ mice showed a significant reduction in active caspase-3 expression relative to littermate controls (Figure 7A), suggesting a reduction of negative selection. Again, no difference was observed for CD8SP thymocytes (Figure 7A). While young B6.AID$^{+/+}$ and B6.AID$^{-/-}$ mice are free of immune infiltration in pancreatic islets, older B6.AID$^{-/-}$ mice display detectable levels of insulitis (Figure 7B), consistent with previously reported insulitis in aged BALB/c.AID$^{-/-}$ mice.[43] In addition, CD4$^{+}$ T cells were detected in the immune infiltrate in those mice, confirming their presence in the insulitic lesions (Figure 7C). Together, these results confirm that the enhanced autoimmunity caused by the inhibition of AID expression by thymic B cells in NOD.AID$^{-/-}$ mice may also impact T-cell tolerance in autoimmune-resistant mice.

## DISCUSSION

In the last few years, thymic B cells have emerged as important mediators of thymic selection.[44,45] Still, the role of thymic B cells in the prevention of T cell-mediated autoimmunity is unclear. In this study, we identified B cell-specific phenotypes in the thymic APC pool of NOD mice, a common mouse model for

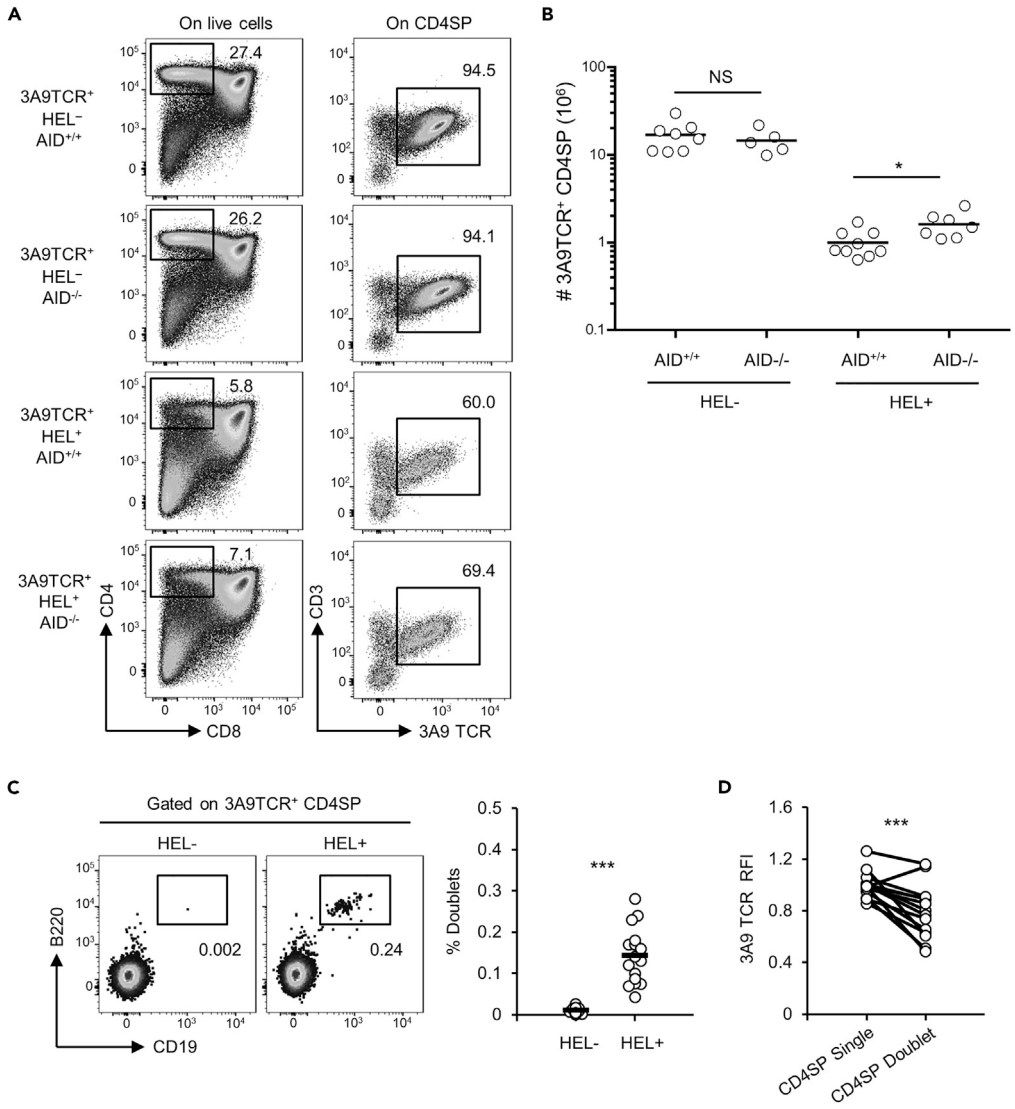


**Figure 6. AID expression promotes clonal deletion of self-reactive thymocytes**

(A) Representative flow cytometry profiles of 3A9 TCR-transgenic CD4SP thymocytes from single transgenic (3A9 TCR$^+$ HEL$^-$) and double transgenic (3A9 TCR$^+$HEL$^+$) B10.BR mice, deficient or not for AID (AID$^{-/-}$ or AID$^{+/+}$ as indicated).

(B) Compilation of the number of 3A9 TCR$^+$ CD4SP thymocytes in the same mice presented in (A) (n = 5-9, Mann-Whitney U test).

(C) Representative flow cytometry profiles (left panel) and compilation (right panel) of conjugates between 3A9 TCR$^+$ CD4SP thymocytes and thymic B cells in HEL$^-$ and HEL$^+$ transgenic B10.BR mice (Mann-Whitney U test).

(D) RFI (Relative fluorescence intensity) of the 3A9 TCR on the surface of 3A9 TCR$^+$ CD4SP thymocytes, conjugated or not with thymic B cells (n = 16, paired t-test). RFI was calculated by normalizing to the average of the 3A9 TCR MFI on CD4SP single cells for each experiment.

The data was acquired in at least three independent experiments. NS, non-significant $p > 0.05$; *, $p < 0.05$; **, ***, $p < 0.001$. Dots represent data for individual mice and the dash represents the mean.

autoimmune diabetes and other autoimmune syndromes.[16,17,46] In addition to a B cell-specific deficit of co-stimulation, NOD thymic B cells do not switch to secondary isotypes as efficiently as in other mouse strains, a trait that correlates with a reduction of CD4SP thymocyte tolerance to self-antigens. To investigate the potential impact of isotype switching by thymic B cells on T-cell tolerance and autoimmunity, we generated NOD.AID$^{-/-}$ mice. As compared to NOD.AID$^{+/+}$ littermates, diabetes was more penetrant in NOD.AID$^{-/-}$ mice, and its onset was accelerated. Adoptive transfer of peripheral T cells and thymocytes revealed an impact of AID expression on T-cell diabetogenicity, consistent with a role for thymic AID expression on

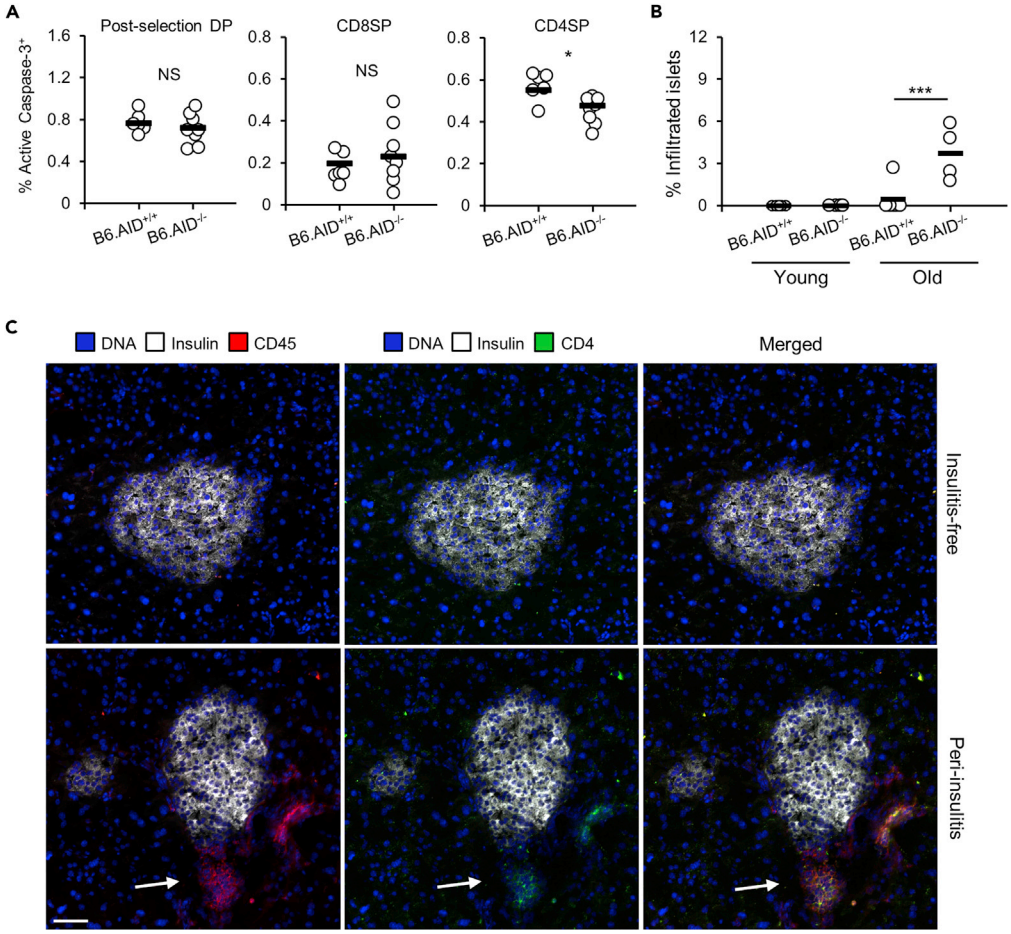


**Figure 7. Deletion of AID in B6 mice limits apoptosis of CD4SP thymocytes and induces insulitis**

(A) Compilation of the percentage of active caspase-3$^{+}$ post-selection DP, CD8SP, and CD4SP thymocytes from B6.AID$^{+/+}$ and B6.AID$^{-/-}$ mice (n = 6-8, Mann-Whitney U test).

(B) Compilation of the percentage of infiltrated pancreatic islets in 2-month-old and 12-month-old B6.AID$^{+/+}$ and B6.AID$^{-/-}$ mice (n = 4-6, Mann-Whitney U test).

(C) Representative fluorescence microscopy images of pancreatic islets from a 12-month-old B6.AID$^{-/-}$ mouse, with (bottom panels) and without (top panels) peri-insulitis (magnification 10X, scale bar represents 50 μm).

The data was acquired in at least three independent experiments. NS, non-significant $p > 0.05$; *, $p < 0.05$; ***, $p < 0.001$. Dots represent data for individual mice and the dash denotes the mean.

thymocyte selection. Negative selection of CD4SP thymocytes was impaired in NOD.AID$^{-/-}$ mice. Using an antigen-specific model of clonal deletion, our data show that AID augments the negative selection of CD4SP thymocytes. Lastly, we find that the deletion of AID in the diabetes-resistant B6 strain resulted in a reduction of CD4SP thymocyte apoptosis and low-level insulitis. Together, these results identify a role for AID expression by thymic B cells in T-cell selection and in the control of autoimmunity.

Previous studies suggested that thymic B cells can directly mediate the negative selection of self-reactive CD4SP thymocytes.[6,7,13] Because of the difficulty of studying negative selection in mice with a polyclonal T-cell and B-cell repertoire, most studies have used mice with expression of TCR and BCR transgenes to investigate the role of thymic B cells in the elimination of autoreactive CD4SP thymocytes.[7,13] For example, thymic B cells express the transcription factor AIRE, enabling expression of tissue-restricted antigens.[7] Although AIRE expression is lower in thymic B cells than in mTECs, its expression is sufficient to drive the negative selection of TCR transgenic CD4SP thymocytes specific for a self-antigen expressed via the AIRE promoter.[7] Similarly, in a BCR and TCR transgenic mouse model, directing BCR specificity toward the same antigen as transgenic thymocytes enabled thymic B cells to support the deletion of the

TCR transgenic thymocytes.[6] Although these studies show that thymic B cells can induce the negative selection of thymocytes when either TCR or BCR specificities are fixed, they do not allow the quantification of the impact of thymic B cells on the negative selection of thymocytes in polyclonal settings. We show that AID expression promotes the negative selection of thymocytes by thymic B cells, even in non-transgenic settings.

First, by comparing thymic B cells from B6 and NOD mice, we find that NOD thymic B cells express low levels of co-stimulatory ligands and exhibit less isotype switching, suggesting that the two phenotypes may be linked. Indeed, weaker thymic B cell activation could result in lower levels of co-stimulation and a decrease in isotype switching. However, the expression of co-stimulatory ligands is not affected by AID disruption. As such, the low level of expression of co-stimulatory ligands on thymic B cells cannot account for the defective tolerance observed in NOD.AID$^{-/-}$ mice. Still, it would be interesting to investigate whether the low expression of co-stimulatory ligands on NOD thymic B cells impacts thymic selection, and, consequently, T-cell tolerance in NOD mice.

By extension, as the absence of AID does not influence the expression of MHC or selected co-stimulatory molecules on thymic B cells, this suggests that AID expression in thymic B cells facilitates the negative selection of CD4SP thymocytes via other means. AID promotes intra-thymic CSR, leading to the expression of IgG or IgA BCRs.[14] We demonstrate that the presence of isotype-switched B cells in the thymus is common across mouse strains. This has also been observed in humans[47,48] and other animals,[49] suggesting an important role of thymic B cell CSR in T-cell development. One hypothesis for the role of isotype-switched B cells in the thymus is that they promote tolerance toward the different immunoglobulin isotypes.[50] While possible for certain isotypes, the scarcity of thymic B cells expressing some antibody subclasses, such as IgG1, IgG3[7] and IgE[29] suggests that this may not be true for all of them. Furthermore, the increase in insulitis caused by AID disruption in this study suggests an impact on tolerance that is broader than against immunoglobulin antigens alone. Analysis of the BCR repertoire of thymic B cells revealed enrichment of self-reactive clones in isotype-switched B cells.[14] Indeed, the thymic environment appears to favor enrichment for self-reactive thymic B cells relative to the periphery.[6] Analysis of B cell specificities in human thymi also reveal enrichment for self-reactive B cells, especially against peptide antigens, including insulin-reactive B cells.[48] Self-reactive thymic B cells may more effectively capture self-antigens through their isotype-switched high-affinity BCRs, allowing enhanced presentation of self-antigens to thymocytes, resulting in greater negative selection. Of interest, among thymic B cells, the frequency of B cells bearing an insulin-specific BCR is reduced in NOD mice relative to B6 mice.[29] This suggests that the reduction in self-reactive B cells in the thymus of NOD mice may explain, to some degree, the impaired tolerance of CD4SP thymocytes to self-antigens. While the factors promoting the accumulation of self-reactive B cells in the thymus are largely unknown, our results suggest that AID expression in thymic B cells enhances their potential to induce negative selection of thymocytes. This may be due, at least in part, to the fact that AID promotes the modification of the BCR through CSR and somatic hypermutation, resulting in the modulation of BCR signaling and antigen specificity.[51–55] Of interest, antibody-secreting plasma cells have also been detected in the thymus of mice.[29,56] While this study focused on the characterization of CD19$^{+}$B220$^{+}$ thymic B cells (i.e., excluding plasma cells), it is possible that the absence of AID may impact thymic plasma cell phenotype or function. More work is needed to understand the role of thymic plasma cells and their secreted antibodies on T-cell tolerance.

As mentioned above, thymic B cells are thought to induce the negative selection of self-reactive CD4SP thymocytes. Negative selection at the CD4SP stage is estimated to take place at a rate of more than $1.6x10^5$ cells per hour in mice, accounting for almost 25% of all clonal deletion.[57] Although there are many APCs in the thymus, we find that thymic B cells play a non-redundant role in the negative selection of self-reactive thymocytes. Using a TCR transgenic system in conjunction with cognate antigen expression in the thymus, we demonstrate that the absence of AID results in an increase in self-reactive CD4SP thymocytes. Of note, this experiment was performed in mice with non-transgenic BCRs, where the B cell repertoire is highly polyclonal. The modest impact on negative selection observed is therefore likely caused by the scarcity of HEL-specific B cells in these mice. Interestingly, the non-redundant role of AID expression in thymic B cells in negative selection is limited to CD4SP thymocytes. Considering that thymic B cells are primarily located in the thymic medulla, it is not surprising that they have a limited impact on DP thymocytes. However, thymic B cells express both MHC I and MHC II. The reason why isotype-switched thymic B cells induce the negative selection of CD4SP thymocyte and not CD8SP thymocytes, is unclear. Still,

our results are consistent with previous reports demonstrating the modulation of CD4SP thymocyte differentiation in B cell-deficient mice, with no overt impact on CD8SP thymocytes.[7]

Various central and peripheral tolerance mechanisms are in place to limit autoimmune diseases. If thymic B cells play a truly non-redundant role in central tolerance, the increase in the number of self-reactive CD4SP thymocytes in AID-deficient mice should increase autoimmune disease susceptibility. Indeed, AID disruption increases autoimmune diabetes incidence. This observation is consistent with a previous report showing that AID deficiency accelerates diabetes onset in NOD mice,[34] although thymic B cells were not studied. While another group observed a reduction of diabetes onset in AID-deficient NOD mice, likely caused by an expansion of regulatory B cells,[58] the diabetogenicity of T cells in AID-deficient NOD mice was increased compared to AID-sufficient NOD mice, consistent with our data and a role for AID expression in thymic B cells in the elimination of pathogenic T cells. We also show that the disruption of AID increases insulitis in diabetes-prone (NOD) and diabetes-resistant (B6) mice. As the nature of the antigens presented by thymic B cells is unknown, it is unclear if antigen presentation by thymic B cells prevents autoimmunity against specific antigens and organs. Interestingly, BALB/c.AID$^{-/-}$ mice display CD4$^{+}$ T cell-mediated infiltration of multiple organs (stomach, liver, lungs salivary glands, and pancreas), consistent with a role for thymic B cells in the elimination of a broad range of self-reactive CD4SP thymocytes. However, this multi-organ immune infiltration may also have been caused by a potential, at the time unknown, mutation in the *Lag3* gene of the BALB/c.AID$^{-/-}$ mice.[59] We have previously reported that B6.AID$^{-/-}$ mice have a reduced lifespan compared to WT littermates,[60] but the cause for this remains unknown. It is tempting to speculate that enhanced autoimmunity caused by loss of AID expression in thymic B cells may contribute to this reduced lifespan. More work is needed to understand the complete impact of the loss of AID expression in thymic B cells in B6 mice.

Mutations in *AID* have been detected in patients with hyper-IgM syndrome.[61] Strikingly, a large portion of those patients present some auto-immune or inflammatory disorders.[62,63] Those include T-cell mediated diseases, such as T1D, Crohn disease and non-infectious uveitis.[62,63] However, as the participation of peripheral B cells has also been reported in those pathologies, we cannot confirm that the increased autoimmunity in AID-deficient patients is due to defects in thymic B cell-mediated T-cell tolerance. Nevertheless, we have shown that increased diabetes onset in NOD.AID$^{-/-}$ mice is, at least in part, T cell-mediated. Furthermore, thymocyte transfers confirm a role for AID expression specifically by thymic B cells in modulating the T-cell repertoire, likely through the promotion of negative selection of CD4SP thymocytes.

In conclusion, this study reveals a non-redundant role for AID expression in thymic B cells in the induction of CD4$^{+}$ T-cell tolerance. Previous studies suggest that isotype-switched thymic B cells have a greater propensity to express a self-reactive BCR, which may facilitate the presentation of self-antigens to thymocytes. Additional studies are required to determine the peptide repertoire presented by isotype-switched thymic B cells, and how this can be manipulated to prevent autoimmune diseases.

## Limitations of the study

In our study, we find that a reduction of CSR in thymic B cells in NOD mice correlates with an impaired tolerance of CD4SP thymocytes to autoantigens. While we confirmed the impact of thymic B cell CSR on T-cell tolerance using NOD.AID$^{-/-}$ mice, other factors in the NOD background likely contribute to the impaired tolerance of CD4SP thymocytes. Also, we focused our study on T-cell tolerance in the context of autoimmune diabetes. The impact of thymic B cells in the prevention of autoimmunity in different contexts, such as autoimmune diseases targeting other organs, remains to be investigated.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- RESOURCE AVAILABILITY
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  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105852.

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## AUTHOR CONTRIBUTIONS

F.L.V. designed and conducted the experiments, prepared the figures, and wrote the article. S.R.T. conducted some experiments. G.C.R. and A.Z. generated and validated the genotype and phenotype of NOD.AID$^{-/-}$ mice and contributed to revisions of the article. J.M.D.N provided some mouse strains, supervised the study, and revised the article. H.J.M. provided reagents, wrote and revised the article. S.L. supervised the study, wrote and revised the article.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| 1G12 | Homemade. See STAR methods for details. | N/A |
| Active Caspase-3 (C92-605) | BD Biosciences | Cat# 559341 |
| B220 (RA3-6B2) | BioLegend | Cat# 103245 |
| CD3 (17A2) | BioLegend | Cat# 100241 |
| CD4 (GK1.5) | Thermo Fisher Scientific | Cat# 48-0041 |
| CD5 (53-7.3) | BioLegend | Cat# 100616 |
| CD8a (53-6.7) | BD Biosciences | Cat# 551162 |
| CD8b (H35-17.2) | Thermo Fisher Scientific | Cat# 11-0083-85 |
| CD11b (M1/70) | BioLegend | Cat# 101226 |
| CD11c (N418) | BioLegend | Cat# 117318 |
| CD19 (6D5) | BioLegend | Cat# 115528 |
| CD40 (FGK4.5) | InVivoMab | Cat# BE0016-2 |
| CD44 (IM7) | BioLegend | Cat# 103030 |
| CD45 (30-F11) | BioLegend | Cat# 103151 |
| CD45.1 (A20) | BioLegend | Cat# 110722 |
| CD45.2 (104) | BioLegend | Cat# 109828 |
| CD62L (MEL-14) | BioLegend | Cat# 104411 |
| CD69 (H1.2F3) | BioLegend | Cat# 104508 |
| CD80 (16-10A1) | BioLegend | Cat# 104708 |
| CD86 (GL-1) | BioLegend | Cat# 105006 |
| CD326/EpCAM (G8.8) | Thermo Fisher Scientific | Cat# 48-5791-82 |
| FOXP3 (FJK-16s) | Thermo Fisher Scientific | Cat# 11-5773 |
| HELIOS (22F6) | Thermo Fisher Scientific | Cat# 17-9883-41 |
| I-$A^d$ (39-10-8): cross-react to I-$A^{g7}$ | BioLegend | Cat# 115009 |
| IA/IE (M5/114.15.2) | BioLegend | Cat# 107626 |
| IgA (RMA-1) | BioLegend | Cat# 407004 |
| IgD (11-26c.2a) | BioLegend | Cat# 405712 |
| IgG1 (RMG1-1) | BioLegend | Cat# 406610 |
| IgG2b (RMG2-1) | BioLegend | Cat# 406706 |
| IgM (RMM1) | BioLegend | Cat# 406513 |
| Insulin (T56-706) | BD Biosciences | Cat# 565689 |
| TCRβ (H57-597) | BioLegend | Cat# 109205 |
| Chemicals, peptides, and recombinant proteins | | |
| Carboxyfluorescein succinimidyl ester (CFSE) | Sigma | Cat# 21888 |
| Collagenase D | Sigma | Cat# 11088866001 |
| DNAse I from bovine pancreas | Sigma | Cat# D4513-1VL |
| Fluoroshield Mounting Medium With DAPI | Abcam | Cat# ab104139 |
| LIVE/DEAD™ Fixable Yellow Dead Cell Stain | Thermo Fisher Scientific | Cat# L34959 |
| LPS | Sigma | Cat# L2654 |
| Papain | Worthington Biochemical | Cat# LS00319 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| RPMI 1640 Medium | Gibco | Cat# 11875-093 |
| Streptavidin | BioLegend | Cat# 405203 |
| **Critical commercial assays** | | |
| Blood glucose meter (Accu-Check Performa) | Roche | N/A |
| Cytofix/Cytoperm | BD Biosciences | Cat# 554714 |
| Diastix | ASCENSIA | Cat# 2806 |
| eBioscience Foxp3/Transcription Factor | Thermo Fisher | Cat# 00-5523-00 |
| **Experimental models: Organisms/strains** | | |
| Mouse: A/J | Jackson Laboratory | #000646 |
| Mouse: C57BL/6 (B6) | Jackson Laboratory | #000664 |
| Mouse: CAST/EiJ (CAST) | Jackson Laboratory | #000928 |
| Mouse: C3H/HeJ (C3H) | Jackson Laboratory | #000659 |
| Mouse: NOD.AID$^{-/-}$ | In-house backcross. See STAR Methods for details | N/A |
| Mouse: NOD/ShiLtJ (NOD) | Jackson Laboratory | #001976 |
| Mouse: NZO/HILtJ (NZO) | Jackson Laboratory | #002105 |
| Mouse: PWK/PhJ (PWK) | Jackson Laboratory | #003715 |
| **Oligonucleotides** | | |
| Primers for AID genotyping | (Muramatsu et al.[64]) | N/A |
| **Software and algorithms** | | |
| Biorender | Biorender | https://biorender.com |
| FlowJo | BD | https://www.flowjo.com |
| GraphPad Prism 7 | GraphPad | https://www.graphpad.com |

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sylvie Lesage (sylvie.lesage@umontreal.ca).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this article is available from the lead contact upon reasonable request.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Mice

A/J (#000646), C57BL/6 (B6, #000664), CAST/EiJ (CAST, #000928), C3H/HeJ (C3H, #000659), NOD/ShiLtJ (NOD, #001976), NZO/HILtJ (NZO, #002105) and PWK/PhJ (PWK. #003715) mice were obtained from Jackson Laboratory (Bar Harbor, United States). B6.AID$^{-/-}$ mice were generously shared by Dr Tasuku Honjo (Kyoto University, Japan). 3A9 TCR transgenic and rat insulin promoter-driven HEL transgenic mice on the B10.BR background were generated by crossing 3A9 TCR transgenic, and ILK3 Ins-HEL transgenic mice to B10.Br/SgSnJ mice.[23] To generate NOD.AID$^{-/-}$ mice, B6.AID$^{-/-}$ mice were backcrossed to NOD mice for 10 generations. Mice were genotyped at each generation, as published,[64] and AID$^{+/-}$ mice

were used for backcrossing to NOD. At the 10$^{th}$ generation, NOD.AID$^{+/-}$ mice were intercrossed to yield NOD.AID$^{-/-}$ as well as NOD.AID$^{+/-}$ and NOD.AID$^{+/+}$ littermates for controls. The genotype of all transgenic mice was verified by PCR. Transgene positive and negative littermates were used in every experiment where applicable. Mice used in experiments were born, housed, and bred in a single room of the specific pathogen-free animal facility at Maisonneuve-Rosemont Hospital. All mice used for experiments were aged between 8-12 weeks, unless indicated otherwise. For phenotyping experiments, both male and female mice were used, with no observed significant differences in phenotype when data was segregated for sex; as such, data from both sexes were pooled. For experiments assessing diabetes onset and insulitis, only female mice were used. Mice were age matched for every experiment. All mouse strains were maintained at the Maisonneuve-Rosemont Hospital animal facility. The Maisonneuve-Rosemont Hospital ethics committee, overseen by the Canadian Council for Animal Protection, approved the breeding of the mice (protocol #2021-2346) and experimental procedures (protocol #2022-2854).

## METHOD DETAILS

### Cell isolation

Thymus, spleen and LNs were pressed through a 70-μm cell strainer (Thermo Fisher Scientific, Waltham, United States). Spleen cell suspensions were treated with $NH_4Cl$ to lyse red blood cells. For TEC analysis, thymi were cut in small pieces and pipetted up and down to release thymocytes. The remaining tissue was digested with papain (0.25 mg/ml), collagenase D (0.25 mg/ml), and DNAse (0.1 mg/ml) for 15 minutes at 37°C in complete RPMI.[65]

### Flow cytometry

Single-cell suspensions were stained for 30 minutes at 4°C with different combinations of antibodies listed in the key resources table. Homemade 1G12 antibodies were used to stain for the 3A9 TCR.[23,66] Biotin-labelled antibodies were revealed with fluorescently-coupled streptavidin. Dead cells were stained using LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit (Thermo Fisher Scientific). For caspase staining, cells were treated with Cytofix/Cytoperm Fixation kit (BD Biosciences) as directed by the manufacturer. The FOXP3 Transcription Factor Staining Buffer Set (eBioscience) was used for transcription factor staining as directed by the manufacturer. Data were collected on an LSRFortessaX20 (BD) and analyzed with FlowJo software (BD Biosciences). B cells were gated as CD19$^+$B220$^+$ cells. TECs, cDC1, cDC2 and pDC were gated as EpCAM$^+$CD45$^-$, CD11c$^{hi}$CD8α$^+$CD11b$^-$, CD11c$^{hi}$CD8$^-$CD11b$^+$, and CD11c$^{low}$B220$^+$, respectively. CD4SP, CD8SP and post-selection DP were gated as TCRβ$^+$CD5$^+$CD4$^+$CD8$^-$, TCRβ$^+$CD5$^+$CD4$^-$CD8$^+$, and TCβ$^+$CD5$^+$CD4$^+$CD8$^+$, respectively.

### *In vitro* activation of thymic B cells

Total thymic cells were incubated for 72h in 96 well plates (flat bottom, 1 x 10$^6$ cells per well) in the presence of LPS (25μg/ml) or anti-CD40 antibodies (10μg/ml). Unstimulated controls were stained directly after mouse sacrifice.

### *In vitro* co-culture

Splenic B cells were positively selected with a magnetic bead isolation kit (STEMCELL technologies, Vancouver, Canada) and anti-CD19 antibodies. B cells were cultured *in vitro* for 3 days with anti-CD40 antibodies (10μg/ml). Activation was confirmed by staining for expression of CD80/CD86 (Figure S1G). CD4SP thymocytes were negatively selected with a magnetic bead isolation kit (STEMCELL technologies) and anti-CD8 and anti-CD19 antibodies. Isolated CD4SP thymocytes were stained with CFSE (Sigma, Saint-Louis, United States) (final concentration of 2 μM). Activated B cells and CFSE-stained CD4SP thymocytes were co-cultured at a 1:1 ratio (2x10$^5$ cells of each per well) in round bottom 96 well plates. Proliferation and activation of thymocytes was assessed by flow cytometry after 72h of co-culture. The proliferation index was measured using FlowJo software.

### Diabetes incidence

Diabetes incidence was monitored daily in female mice for overt signs of diabetes (wet cage, hunched posture) and every 2 weeks for urine glucose levels using Diastix (Bayer, Toronto, Canada) starting before the age of 10 weeks. After a positive Diastix test, overt diabetes was confirmed by blood glucose levels > 12 mmol/L, measured using Accu-Chek strips (Roche, Basel, Switzerland). The mice were sacrificed

within 1 week of detection of high blood glucose or when they reached > 34 weeks of age. The pancreas was collected and conserved in formalin for at least 48h before paraffin embedding.

### Histology

Hematoxylin and eosin staining was performed on 6 μm pancreas sections from paraffin blocks, for 2 non-successive sections per slide with 2 slides per mouse. Slides were scored for infiltration according to the following scale: 0 = no infiltration, 1 = peri-insulitis, 2 = infiltration <50%, 3 = infiltration >50%, 4 = complete infiltration.[67]

### Fluorescence microscopy

Pancreas were frozen in OCT (Thermo Fischer Scientific) over dry ice, and 10 μm slices of tissues were prepared using a cryostat. Slides were washed for 5 minutes in PBS (Wisent Bio, Saint-Jean-Baptiste, Canada) three times and fixed with 3.7% paraformaldehyde (Sigma) for 10 minutes. Slides were washed again in PBS three times, permeabilized, and blocked for 60 minutes using TritonX100 (0.1%) and BSA (3% in PBS). Antibodies (FITC-labelled anti-CD4, PE-labelled anti-CD45, Alexa Fluor 647-labelled anti-insulin) were added and incubated for 2 hours at room temperature then washed in PBS three times. Mounting medium containing DAPI (Abcam, Cambridge, United Kingdom) was added, and slides were sealed with nail polish. Images were acquired using a fluorescent microscope (Axio Imager 2, ZEISS).

## QUANTIFICATION AND STATISTICAL ANALYSES

Data were tested for significance using a nonparametric Mann-Whitney U test, a one-way ANOVA, or a paired t-test. A Log-Rank (Mantel Cox) test was used for the incidence studies. Numbers of animals used per group (n) are indicated in the figure legends. The minimal significance threshold was set at 0.05 for all tests. NS, non-significant $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.