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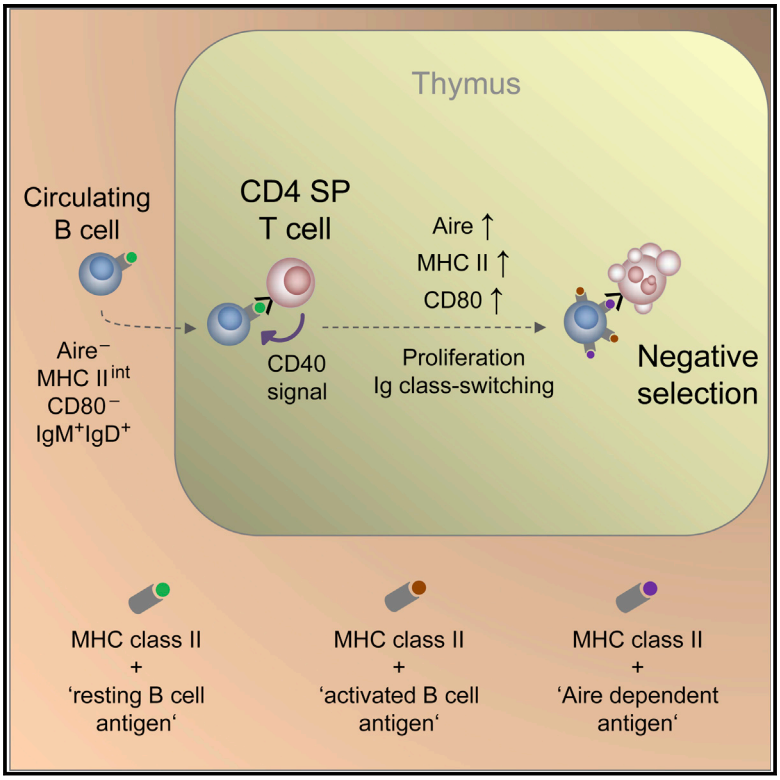
Immunity

Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction

Graphical Abstract



Highlights

- Circulating naive B cells immigrate into the thymus
- They receive CD40 signals in the context of cognate interactions with $CD4^+$ thymocytes
- This licenses B cells to express Aire and upregulate MHC class II and CD80
- Thymic B cells present a licensing-dependent endogenous self-antigen for negative selection

Authors

Tomoyoshi Yamano, Jelena Nedjic, Maria Hinterberger, ..., Benedikt Brors, Bruno Kyewski, Ludger Klein

Correspondence

ludger.klein@med.lmu.de

In Brief

Thymic dendritic cells and medullary epithelial cells mediate central tolerance, but whether also thymic B cells harbor distinct tolerogenic features is unclear. Klein and colleagues demonstrate that B cells migrating into the thymus undergo licensing for self-antigen presentation, which contributes to T cell negative selection.

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Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction

Tomoyoshi Yamano,[1] Jelena Nedjic,[1] Maria Hinterberger,[1] Madlen Steinert,[1] Sandra Koser,[2] Sheena Pinto,[3] Norbert Gerdes,[4] Esther Lutgens,[4,5] Naozumi Ishimaru,[6] Meinrad Busslinger,[7] Benedikt Brors,[2] Bruno Kyewski,[3] and Ludger Klein[1,*]

[1]Institute for Immunology, Ludwig-Maximilians-University Munich, Goethestrasse 31, 80336 Munich, Germany
[2]Division of Theoretical Bioinformatics, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
[3]Division of Developmental Immunology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
[4]Institute for Cardiovascular Prevention, Ludwig-Maximilians-University Munich, Pettenkoferstrasse 9, 80336 Munich, Germany
[5]Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 Amsterdam, the Netherlands
[6]Department of Oral Molecular Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8504, Japan
[7]Research Institute of Molecular Pathology, Vienna Biocenter, Dr. Bohr-Gasse 7, 1030 Vienna, Austria
*Correspondence: ludger.klein@med.lmu.de
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SUMMARY

Thymic antigen-presenting cells (APCs) such as dendritic cells and medullary thymic epithelial cells (mTECs) use distinct strategies of self-antigen expression and presentation to mediate central tolerance. The thymus also harbors B cells; whether they also display unique tolerogenic features and how they genealogically relate to peripheral B cells is unclear. Here, we found that Aire is expressed in thymic but not peripheral B cells. Aire expression in thymic B cells coincided with major histocompatibility class II (MHCII) and CD80 upregulation and immunoglobulin class-switching. These features were recapitulated upon immigration of naive peripheral B cells into the thymus, whereby this intrathymic licensing required CD40 signaling in the context of cognate interactions with autoreactive $CD4^+$ thymocytes. Moreover, a licensing-dependent neo-antigen selectively upregulated in immigrating B cells mediated negative selection through direct presentation. Thus, autoreactivity within the nascent T cell repertoire fuels a feed forward loop that endows thymic B cells with tolerogenic features.

INTRODUCTION

Various thymic stromal cell types together establish a unique microenvironment for the generation of a functional and self-tolerant T cell repertoire (Klein et al., 2014). Individual stromal cell subsets are positioned in distinct anatomical niches and exhibit functional adaptations allowing them to support particular aspects of T cell differentiation. Cortical thymic epithelial cells (cTECs) serve an indispensable role in positive selection, specified in part by unique proteolytic pathways related to antigen presentation (Guerder et al., 2012; Klein et al., 2014; Takahama et al., 2012). Medullary thymic epithelial cells (mTECs), through promiscuous expression of tissue-restricted antigens (TRAs) and direct presentation of these TRAs, fulfill a dual role as self-antigen reservoir and antigen-presenting cells (APCs) (Derbinski et al., 2001; Hinterberger et al., 2010). Thymic classical dendritic cells (cDCs) comprise intrathymically—and peripherally—derived subsets (Wu and Shortman, 2005). cDCs are commonly considered the major hematopoietic APC type involved in central tolerance, owing to their efficacy in capturing and presenting serum-borne, mTEC-derived, or peripherally acquired self-determinants (Atibalentja et al., 2009; Bonasio et al., 2006; Koble and Kyewski, 2009).

The thymus also harbors stromal cells whose contribution to T cell selection is less well understood. Macrophages have a crucial function in the removal of apoptotic thymocytes, whereas their role as bona fide APCs has remained contentious (Volkmann et al., 1997). Plasmacytoid DCs were shown to import peripherally acquired antigens into the thymus and present these for central tolerance induction (Hadeiba et al., 2012). The thymus also contains B cells, whose capacity to act as APCs for central tolerance has been documented in the context of superantigens or B cell receptor (BCR)-mediated antigen capture and presentation, through B-cell-specific expression of a self-antigen or by selectively expressing major histocompatibility complex (MHC) molecules on B cells (Frommer and Waisman, 2010; Fujihara et al., 2014; Kleindienst et al., 2000; Perera et al., 2013). Recent evidence also suggests that thymic B cells might be involved in regulatory T cell induction (Walters et al., 2014). However, it remains to be established whether thymic B cells display unique tolerogenic features distinguishing them from peripheral B cells. Moreover, their precise genealogical relationship to B cells in secondary lymphoid organs is unknown.

Among the various functional adaptations of thymic APCs for T cell repertoire selection, promiscuous gene expression stands out owing to its link to the monogenically inherited human autoimmune disease APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy). Mutations in *AIRE* (autoimmune regulator) are the causal defect in APECED, and mice

deficient in Aire recapitulate several features of APECED (Mathis and Benoist, 2009; Peterson et al., 2008). TRA expression is substantially reduced in *Aire*$^{-/-}$ mTECs, and there have been recent advances in deciphering the molecular workings of Aire (Abramson et al., 2010; Anderson et al., 2002; Mathis and Benoist, 2009; Peterson et al., 2008). Besides its function in the promotion of promiscuous gene expression, Aire has also been implicated in mTEC differentiation, chemokine production, and thymic DC positioning, and fate-mapping studies insinuated extrathymic functions during early embryogenesis (Laan et al., 2009; Lei et al., 2011; Nishikawa et al., 2010; Yano et al., 2008). Moreover, there is accruing evidence for Aire expression in cells other than mTECs (Gardner et al., 2009), and a role of Aire beyond mTECs is strongly supported by the detection of Aire expression in a rare cell population in lymph nodes and spleen (Gardner et al., 2008). These extrathymic Aire-expressing cells (eTACs) express Aire-regulated TRAs distinct from those in mTECs, are of hematopoietic origin and exhibit certain DC features; however, their exact lineage identity remains elusive (Gardner et al., 2013). The present study was initiated to address the possibility that also within the thymic microenvironment, the expression and function of Aire might not be solely restricted to mTECs.

RESULTS

Aire Is Expressed in Thymic but Not Peripheral B Cells

Aire-HCO mice carry a transgenic *Aire* gene locus encoding a chimeric influenza hemagglutinin (HA) protein and a human (h) *CD2* reporter (Aschenbrenner et al., 2007; Hinterberger et al., 2010). Besides the expected Aire-reporter-expressing EpCAM^{+} mature mTECs, we noted a population of non-epithelial (EpCAM^{-}) reporter^{+} cells in thymi from both Aire-HCO and Adig Aire-GFP-reporter mice (Figure 1A; Gardner et al., 2008). These EpCAM^{-}reporter^{+} cells were similarly found in Adig → WT bone marrow (BM) chimeras (Figure 1B), indicating their hematopoietic origin and excluding false positives emerging from intercellular transfer of mTEC-derived proteins (Koble and Kyewski, 2009). Around 95% of these EpCAM^{-}Aire-reporter^{+} cells expressed CD19, and a minor fraction expressed CD11c (Figure 1B).

CD19^{+} cells account for 0.2%–0.5% of the thymic cellularity (Figure S1A; Akashi et al., 2000; Perera et al., 2013). In BM chimeras, we consistently observed a 4- to 5-fold reduction of thymic CD19^{+} cells (Figure S1A), matching the reduction of reporter^{+} cells observed in Adig → WT bone marrow (BM) chimeras compared to unmanipulated Adig mice (Figures 1A and 1B). Essentially all thymic CD19^{+} cells expressed a *Pax5* reporter (Figure S1B). Moreover, EpCAM^{-}Aire-reporter^{+} cells were largely absent from thymi of B-cell-deficient homozygous *Mb1*-Cre$^{knockin/knockin}$ (Hobeika et al., 2006) Aire-reporter mice (referred to as *Mb1*-Cre$^{ki/ki}$), confirming their B cell identity (Figure 1C). Remarkably, among thymic CD45^{+} MHCII^{+} cells, B cells were at least as abundant as CD11c^{+} DCs (Figure S1C).

Of thymic B cells, around 50% were Aire-reporter^{+}, whereas reporter expression was undetectable in B cells from spleen or BM (Figure 1D). In further distinction from B cells in other organs, thymic B cells were homogenously MHCII^{hi} and CD80^{+} (Figure 1E; Perera et al., 2013). Endogenous Aire mRNA was readily detectable in reporter^{+} thymic B cells, confirming faithful expression of the reporter (Figure 1F). Intracellular staining revealed Aire protein in reporter^{+} but not reporter^{-} thymic B cells, albeit at substantially lower amounts compared to Aire-reporter^{+} mTECs (Figure 1G). Imaging flow cytometry revealed that Aire in thymic B cells localized to one or few nuclear dots that were reminiscent of Aire's sub-cellular localization in mTECs (Su et al., 2008), yet less numerous (Figures 1H, S1D, and S1E). Of thymic B cells, 2.2% ± 0.1% and of mTECs 26% ± 3.5% displayed a punctate, nuclear distribution of Aire protein (Figures S1D and S1E).

Gene expression profiles of thymic B cells from *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice revealed 205 significantly differentially expressed transcripts. This is in a similar range as what has been observed for eTACs (Gardner et al., 2008), whereas several thousand genes have been estimated to be modulated by Aire in mTECs (Anderson et al., 2002; Derbinski et al., 2005; Sansom et al., 2014). Of the differentially expressed transcripts in thymic B cells, 88 were *Aire* induced and 117 were *Aire* repressed (Tables S1 and S2). Among *Aire*-induced transcripts, 21 (23.9%) were classified as TRAs according to previously used criteria and manual scoring (Table S1; Derbinski et al., 2005). Thus, unlike the reported Aire-dependent transcriptomes of mTECs or eTACs (Anderson et al., 2002; Derbinski et al., 2005; Gardner et al., 2008), Aire-induced genes in thymic B cells are not enriched in TRAs compared to any random set of genes (around 25%) (Anderson et al., 2002; Gardner et al., 2008).

Expression analyses of representative TRAs (*Grik2*, expressed in the brain; *Ggn*, spermatocytes; *Lamp3*, lung) confirmed that their mRNA levels correlated with the expression of Aire (Figure 1H). Strikingly, these transcripts were only weakly expressed in mTECs (Figure 1H). Moreover, the overlap between Aire-induced transcripts in thymic B cells and previously reported Aire-dependent genes in mTECs or eTACs was limited to one or two genes, respectively (Table S1).

Together, these data identified Aire expression as a surprising characteristic of thymic B cells. This unique feature of thymic B cells induced a distinct transcriptional program and went along with the display of potent APC surface characteristics.

Intrathymic Licensing of Immigrating B Cells

Based upon previous reports on intrathymic B lymphopoiesis (Akashi et al., 2000), we considered that Aire expression might be an intrinsically programmed feature of an intrathymically differentiating B lineage. Around one third of CD19^{+} thymic B cells were surface IgM^{-}IgD^{-} and thereby resembled B cell precursors in the BM (Figure 2A). However, whereas in the BM CD19^{+}IgM^{-}IgD^{-}cells could be subdivided into CD2^{+}c-Kit^{-} pre-B cells and CD2^{-}c-Kit^{+} pro-B cells, CD2^{-}c-Kit^{+} pro-B cells and transitional CD2^{-}c-Kit^{-} cells were essentially undetectable in the thymus. Moreover, most thymic CD19^{+}IgM^{-}IgD^{-} cells, in distinction from genuine BM pre-B cells, expressed isotype-switched surface (s)Igs, suggesting that they were the progeny rather than the precursors of IgM^{+}IgD^{+} and IgM^{+}IgD^{-} cells (Figures 2A and S2). This paucity of B cell precursors rendered intrathymic B lymphopoiesis an unlikely source of the majority of thymic B cells.

Compared to IgM^{-}IgD^{-} thymic B cells (most of which had class-switched) and IgM^{+}IgD^{-} cells, Aire-reporter^{+} cells were

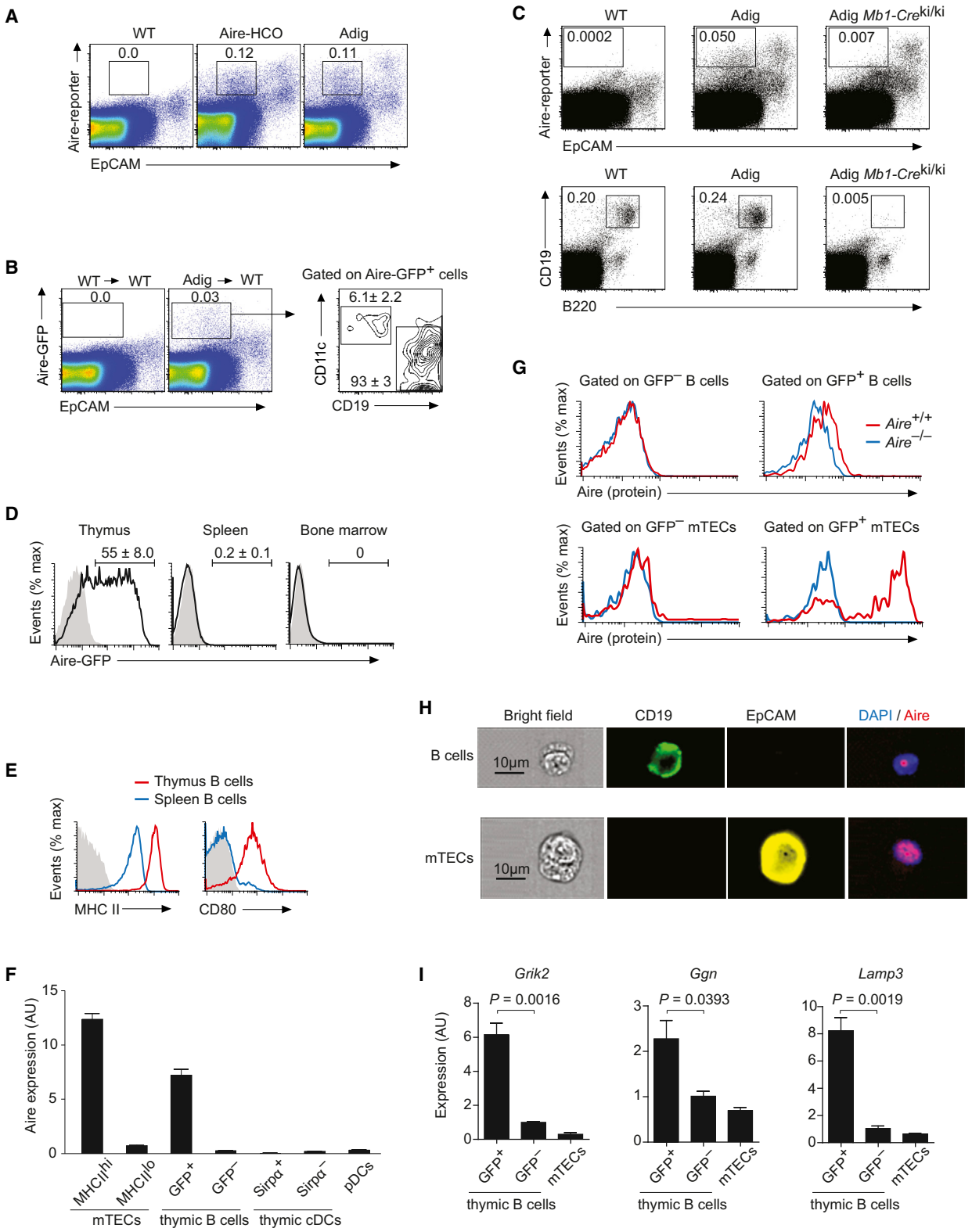

Figure 1. Thymic B Cells Express Aire and Display Enhanced APC Features

(A) Expression of Aire reporters (hCD2 or GFP) in total thymus cells from Aire-HCO or Adig mice, respectively. The average frequency of Aire-reporter$^+$EpCAM$^-$ cells is indicated (n = 4 each).

(B) Expression of Aire-GFP in thymus cells from WT → WT and Adig → WT BM chimeras. The dot plot on the right shows CD19 versus CD11c on gated Aire-GFP$^+$ cells (n = 4 each).

(legend continued on next page)

less frequent among thymic IgM$^+$IgD$^+$ B cells, i.e., among cells resembling naive peripheral B cells (Figure 2B). This raised the possibility that Aire expression and other distinctive features of thymic B cells might be induced in circulating peripheral B cells subsequent to entry into the thymus. We therefore tested whether thymic immigration of peripheral B cells occurred and whether it coincided with the acquisition of steady-state thymic B cell characteristics. Seven days after intravenous transfer of splenic B cells, donor B cells were detectable in the thymus (Figure 2C). Essentially all of these cells exhibited elevated MHCII, and around 50% had become Aire-reporter positive, whereas donor cells in the spleen retained their initial Aire$^-$MHCII^{int} phenotype. Donor B cells in the thymus underwent several divisions, whereby Aire and surface Ig expression correlated with the extent of proliferation (Figure 2D). To more rigorously test whether these phenotypic changes reflected an intrathymically induced process and to also exclude expansion or accumulation of pre-existing Aire-GFP$^+$ and/or sIgG$^+$ cells, we performed intrathymic injections of purified Aire-GFP$^-$IgD$^+$IgG$^-$ lymph node B cells, which confirmed the efficient induction of Aire expression and class switching within the thymus (Figure 2E).

Thus, thymic homing of mature peripheral B cells induces phenotypic changes recapitulating several hallmarks of steady-state thymic B cells. We will refer to this microenvironmental programming as thymic B cell licensing.

B Cell Licensing Requires CD40 Signaling

Thymic B cell licensing resembled the phenotypic changes that accompany the process of mTEC maturation (MHCII^{lo}CD80$^-$ Aire$^-$ → MHCII^{hi}CD80$^+$Aire$^+$), which is primarily orchestrated by receptor activator of NF-κB (Rank) signaling (Akiyama et al., 2008; Hikosaka et al., 2008; Rossi et al., 2007). However, whereas immature mTECs cultured in the presence of Rank-ligand expressing stromal cells expectedly initiated Aire expression, B cells treated in the same manner or with an agonistic Rank antibody remained Aire$^-$MHCII^{int} (Figures 3A and S3). By contrast, an agonistic anti-CD40 antibody induced Aire-reporter expression and Aire mRNA transcription together with MHCII up-regulation in B cells (Figures 3A, 3B, and S3). In *Cd40*$^{-/-}$ and *Cd40lg*$^{-/-}$ mice, thymic but not peripheral B cells were substantially diminished, consistent with CD40 signals controlling thymic B cell numbers (Figure 3C; Fujihara et al., 2014). Most significantly, thymic B cells in these mice did not express Aire mRNA, had not undergone class-switching, and failed to upregulate CD80 (Figures 3D–3F). Thus, distinct signals control the acquisition of a MHCII^{hi}CD80$^+$Aire$^+$ phenotype by mTECs or licensed thymic B cells.

Thymic B Cell Licensing Requires Cross-Talk with CD4 Single-Positive Cells

We next explored from which source the signals for B cell licensing emanated. Among thymocyte subsets, only CD4 single-positive (SP) cells had detectable CD40L on their surface (Figure S4A; Hikosaka et al., 2008; Irla et al., 2008). Thymic B cells in *Tcra*$^{-/-}$ mice, where thymocyte development is arrested at the CD4$^+$CD8$^+$ stage, were Aire negative and had not upregulated MHCII, consistent with a critical role of SP thymocyte-derived signals (Figures 4A and 4B). By contrast, Aire$^+$ mTECs were only quantitatively reduced in these mice, further corroborating the idea that distinct signals orchestrate B cell licensing and mTEC maturation. Adoptively transferred B cells homing to the thymus of *Ciita*$^{-/-}$ recipients, which lack CD4 SP cells, failed to upregulate Aire and MHCII, confirming that B cell licensing crucially required CD4 SP cells (Figure S4B).

Surprisingly, although crossing an OVA-specific TCR transgene (OTII) into the *Tcra*$^{-/-}$ strain rescued a sizable CD4 SP compartment, thymic B cells in these mice neither expressed Aire nor up-regulated MHCII (Figure 4C, compare Figure 4A). We considered whether a lack of CD40L on monoclonal anti-foreign CD4 SP thymocytes might account for this. However, their CD40L expression was similar to that of polyclonal CD4 SP cells, indicating that CD40L on CD4 SP cells is independent of agonist autoreactive TCR stimulation, yet probably reflects the preceding positively selecting weak interactions with cTECs (Figure S4C; Irla et al., 2008). A significant, albeit slight, restoration of B cell licensing was seen in OTII *Tcra*$^{+/-}$ mice (Figures 4C and 4D), in which endogenous TCRα rearrangements moderately diversify the TCR repertoire. Thus, B cell licensing crucially involves signals from CD4 SP cells and requires a degree of diversity among TCR specificities.

Licensing Is Initiated through BCR-Independent Self-Antigen Presentation

We hypothesized that the requirement for diverse TCRs reflected a critical role of autoreactivity within the nascent CD4 SP compartment. In *Ciita*$^{-/-}$ → WT BM chimeras, all hematopoietic APCs lack MHCII, and impaired negative selection results in an enlarged CD4 SP compartment (Figure S5A; Hinterberger et al., 2010; van Meerwijk et al., 1997). Unexpectedly, despite this increase in CD4 SP cell autoreactivity, thymic B cells did not undergo licensing (Figure S5B). Because B cells themselves lack MHCII in such chimeras, a plausible explanation was that licensing-inducing CD4 SP cell-B cell interactions did not occur in a bystander fashion, but required direct MHCII-restricted antigen presentation by immigrating B cells. Indeed, adoptively transferred MHCII-less (*Ciita*$^{-/-}$) B cells did not upregulate Aire

(C) EpCAM$^-$Aire-GFP$^+$ cells are largely absent in B-cell-deficient (*Mb1*-Cre$^{ki/ki}$) Adig mice. The upper dot plots show the expression of GFP in total thymus cells from WT controls, Adig, or Adig (*Mb1*-Cre$^{ki/ki}$) mice. The lower dot plots show staining for CD19 and B220 in total thymus cells from WT controls, Adig, or Adig (*Mb1*-Cre$^{ki/ki}$) mice (representative of n ≥ 3 each).
(D) Expression of Aire-GFP in CD19$^+$B220$^+$ cells from thymus, spleen, or BM of Adig mice (n = 4).
(E) MHCII and CD80 on B cells from thymus (red) or spleen (blue).
(F) Aire mRNA in thymic APC subsets. Data show mean values ± SEM of triplicates (AU; arbitrary units).
(G) Intracellular staining for Aire protein in Aire-GFP$^-$ or Aire-GFP$^+$ thymic B cells (top) or mTECs (bottom) from Adig *Aire*$^{+/+}$ mice (red) or Adig *Aire*$^{-/-}$ controls (blue).
(H) Nuclear localization of Aire protein in thymic B cells or mTECs visualized by imaging flow cytometry (see also Figure S1).
(I) Relative mRNA expression of TRAs in Aire-GFP$^+$ B cells, Aire-GFP$^-$ B cells, and mTECs.
Data show the mean ± SEM from three biological replicates (AU; arbitrary units). See also Figure S1 and Tables S1 and S2.

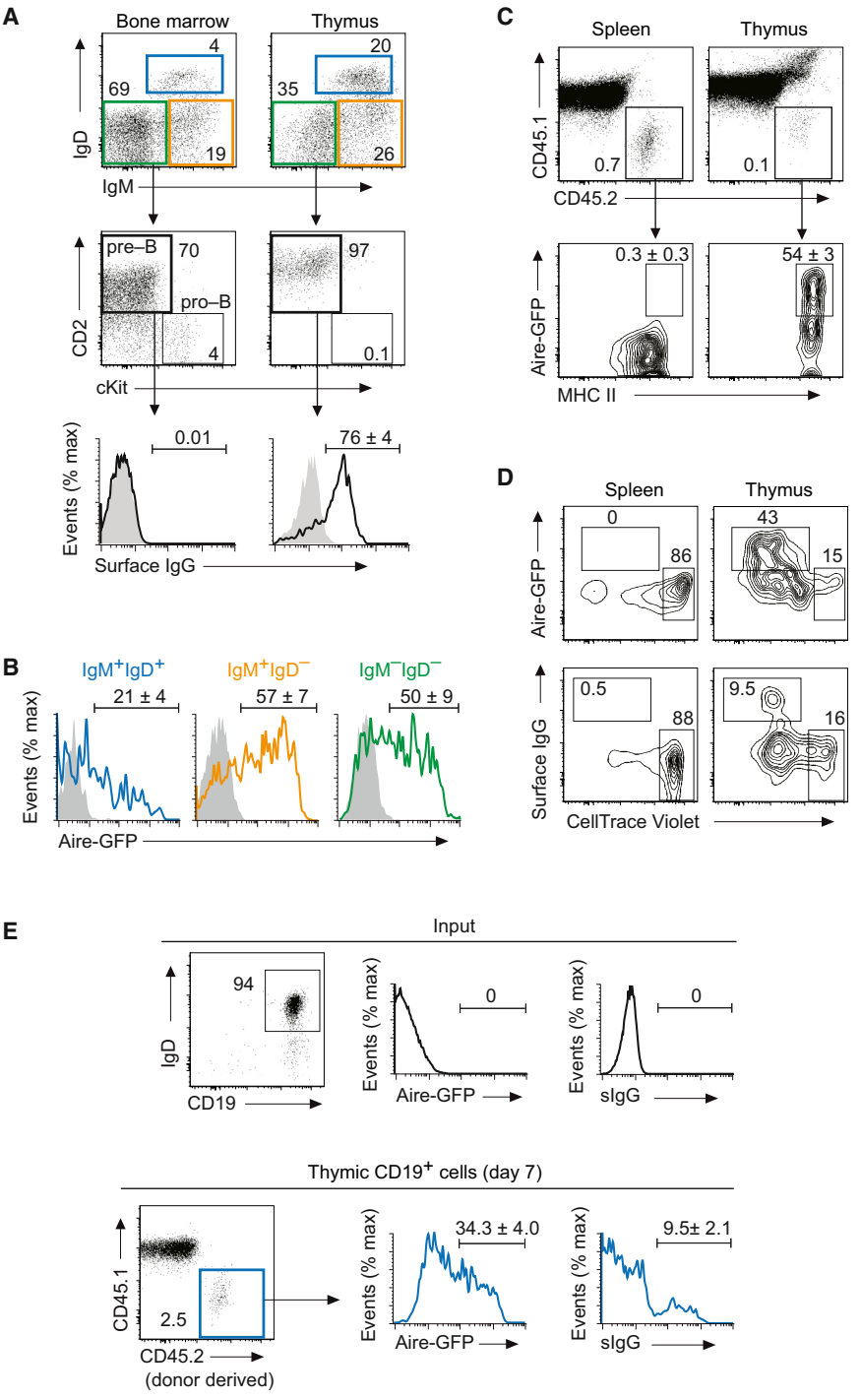

Figure 2. Immigrating B Cells Adopt the Licensed Phenotype of Steady-State Thymic B Cells

(A) Top: Surface IgD and IgM on CD19$^+$ B cells in BM or thymus. Middle: CD2 versus cKit on CD19$^+$IgM$^-$IgD$^-$ B cells from the BM or thymus. Bottom: Surface (s)IgG on CD19$^+$IgM$^-$IgD$^-$CD2$^+$ B cells (n = 3).

(B) Expression ± SEM of Aire-GFP in IgM$^+$IgD$^+$, IgM$^+$IgD$^-$, and IgM$^-$IgD$^-$ thymic B cells from Adig mice, gated as in (A) (n = 4).

(C) Frequency of donor cells and expression of MHCII and Aire-GFP among CD19$^+$ B cells in spleen and thymus of CD45.1 mice 7 days after i.v. injection of splenic B cells from CD45.2 Adig mice (n = 4). The mean fluorescence intensity (MFI) ± SEM of MHCII was 9,600 ± 1,000 in the spleen and 31,000 ± 6,300 in the thymus (p = 0.0006).

(D) Aire-GFP or surface IgG expression in donor B cells in spleen and thymus 7 days after transfer of CellTrace-Violet-labeled splenic B cells from CD45.2 Adig mice into CD45.1 recipients (n = 3).

(E) Purified CD19$^+$sIgD$^+$ lymph node B cells undergo licensing upon intrathymic injection. Top: Reanalysis of sorted follicular B cells from CD45.2 Adig mice before intrathymic injection into CD45.1 recipients. Bottom: Percentage of donor cells (CD45.2$^+$) among thymic CD19$^+$ B cells and expression of Aire-GFP and surface (s)IgG 7 days after i.t. injection (n = 4).

See also Figure S2.

upon entering the thymus (Figure S5C). Most importantly, MHCII-less thymic B cells in 1:1 mixed [*Ciita*$^{-/-}$: WT] → WT BM chimeras, in contrast to MHCII-sufficient B cells co-existing in the identical microenvironment, failed to display any manifestation of licensing, conclusively documenting a B-cell-autonomous requirement for MHCII (Figure 5A). Furthermore, in vitro co-culture with DO11.10 *Rag2*$^{-/-}$ CD4 SP cells triggered Aire expression in peripheral B cells when these presented the OVA-peptide recognized by the DO11.10 TCR (Figure 5B), consistent with MHCII-restricted cognate B cell-CD4 SP cell interactions being a platform for CD40 signaling for B cell licensing.

B cells are comparably inefficient in MHCII-restricted presentation of exogenous substrates, whereas BCR-mediated antigen uptake leads to exceptionally efficient presentation (Lanzavecchia, 1985; Yuseff et al., 2013). Hence, we wondered whether natural autoreactivity within the polyclonal B cell repertoire (Wardemann et al., 2003) might facilitate BCR-mediated self-antigen presentation, which in turn promotes licensing. If this were the case, non-autoreactive B cells would be disfavored or even excluded from this process. We tested this idea with SW_{HEL} mice, which express a transgenic anti-foreign BCR specific for hen egg lysozyme (HEL) on the majority of B cells (Phan et al., 2003). HEL-specific B cells can be distinguished from cells displaying endogenously rearranged diverse BCRs (likely harboring a similar degree of autoreactivity as the polyclonal BCR repertoire) by incubation with HEL followed by staining with an anti-HEL antibody. The percentage of HEL-specific cells among thymic B cells of SW_{HEL} mice was similar to that in the spleen (Figure 5C). Moreover, these HEL$^+$ cells expressed Aire (Figure 5D), had elevated MHCII and CD80, and underwent class-switching (Figure 5E) to an extent that closely resembled or

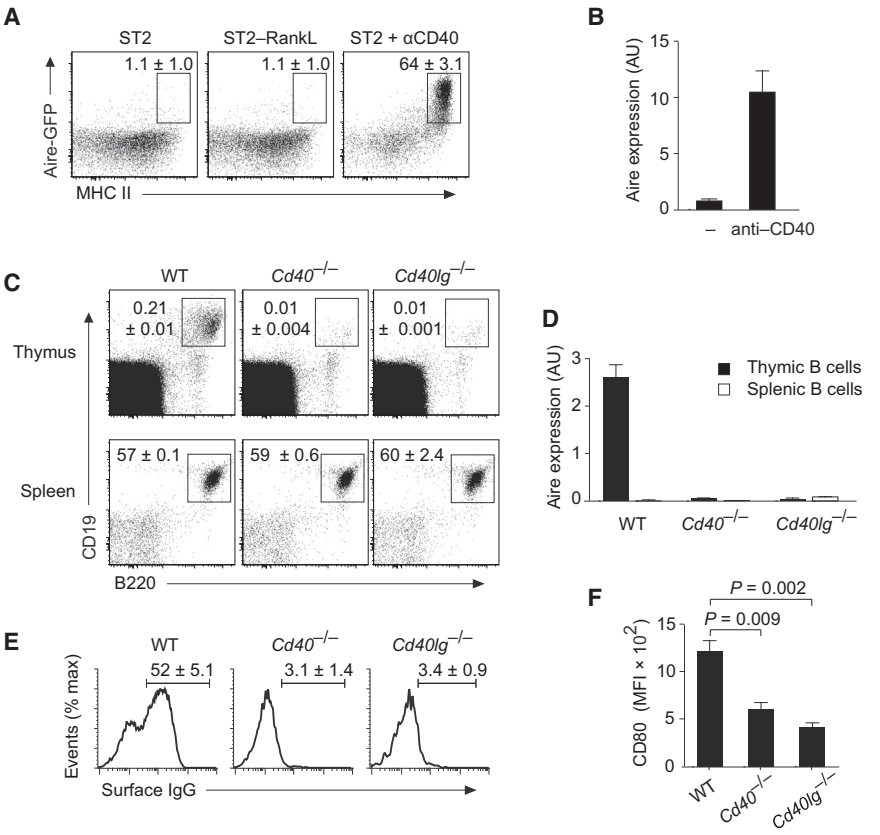

Figure 3. CD40 Signaling Is Sufficient and Necessary for B Cell Licensing

(A) MHCII and Aire-GFP in Adig BM B cells after culture for 3 days with ST2 stromal cells with or without agonistic anti-CD40 antibody or with ST2-RankL cells (n = 4).

(B) Aire mRNA in B cells cultured for 3 days with or without agonistic anti-CD40. Mean ± SEM of three replicates.

(C) CD19 and B220 on thymus or spleen cells from WT, *Cd40*$^{-/-}$, or *Cd40lg*$^{-/-}$ mice.

(D) Aire mRNA in thymic or splenic B cells from WT, *Cd40*$^{-/-}$, or *Cd40lg*$^{-/-}$ mice. Mean ± SEM of three replicates.

(E) Surface IgG on thymic B cells from WT, *Cd40*$^{-/-}$, or *Cd40lg*$^{-/-}$ mice.

(F) CD80 MFI on thymic B cells from WT, *Cd40*$^{-/-}$, or *Cd40lg*$^{-/-}$ mice.

Data in (C), (E), and (F) are from n = 3. See also Figure S3.

even exceeded the respective hallmarks of licensing in cells expressing diverse BCRs (HEL-BCR^{-}). Thus, BCR autoreactivity was a prerequisite neither for B cell immigration into the thymus nor for subsequent licensing.

Next, we addressed whether expression of Aire affected other manifestations of thymic B cell licensing in a cell-intrinsic manner. To this end, we generated mixed [*Aire*$^{-/-}$: WT] → WT BM chimeras. Strikingly, compared to their WT counterparts, Aire-deficient thymic B cells in these chimeras consistently displayed a diminished extent of MHCII upregulation and Ig class-switching (Figure 5F). Together, these data showed that BCR-independent, MHCII-restricted presentation of self to CD4 SP cells establishes licensing-inducing B cell-T cell interactions. Moreover, Aire influences B cell licensing in a cell-intrinsic manner, possibly through a feed forward loop.

BCR Stimulation Counteracts Aire Induction

Several aspects of thymic B cell licensing (the initiating cognate B cell-T cell interplay, the central role of CD40, MHCII and CD80 up-regulation, and class-switching) were reminiscent of the germinal center (GC) reaction in secondary lymphoid tissues (Victora and Nussenzweig, 2012). Indeed, around 20% of thymic B cells displayed a GC-like Fas^{+}GL7^{+} phenotype (Figure 6A). We therefore wondered whether induction of Aire also occurred in peripheral GC B cells. However, neither steady-state Fas^{+}GL7^{+} B cells in spleen, lymph nodes, or Peyer's patches of untreated mice nor GC B cells induced by immunization expressed Aire (Figures 6A and 6B).

Given that despite the obvious parallels between thymic licensing and the GC reaction, GC B cells do not express Aire, we presumed that either a unique CD4 SP cell-derived co-signal other than CD40L is necessary for Aire expression during thymic licensing or that a distinct GC-associated co-signal prevents Aire expression in GC B cells. Our previous observations indicated that thymic B cell licensing in all likelihood occurred independently of BCR stimulation, which contrasts with a central hallmark of B cell activation during GC formation. We therefore tested whether Aire induction in B cells through CD40 stimulation in vitro was influenced by concomitant BCR activation. Indeed, cross-linking of the BCR simultaneous to CD40 stimulation strongly diminished the upregulation of Aire in B cells, whereas it had a synergistic effect in enhancing MHCII expression (Figure 6C). These findings suggest that lack of BCR triggering mechanistically separates thymic B cell licensing from B cell activation in GCs.

Direct Presentation of a Licensing-Associated Neoantigen by Thymic B Cells

Mice lacking B cells (*Mb1*-Cre$^{ki/ki}$) (Hobeika et al., 2006) displayed a significantly enlarged thymic CD4 SP cell compartment (Figure 7A). This was reminiscent of previous observations in mice that either lacked DCs or had a diminution of MHCII on mTECs, suggesting a non-redundant contribution of thymic B cells to negative selection (compare also Figure S5A; Birnberg et al., 2008; Hinterberger et al., 2010; Ohnmacht et al., 2009). CD4^{+} T cells from B-cell-deficient mice proliferated more than control B cells upon transfer into syngenic recipients, and a considerable fraction of these cells spontaneously acquired a CXCR5^{+}PD-1^{+} follicular T helper cell phenotype (Figure 7B). Although this was consistent with a distinct contribution of B cells to CD4 T cell tolerance, it failed to distinguish between central and peripheral

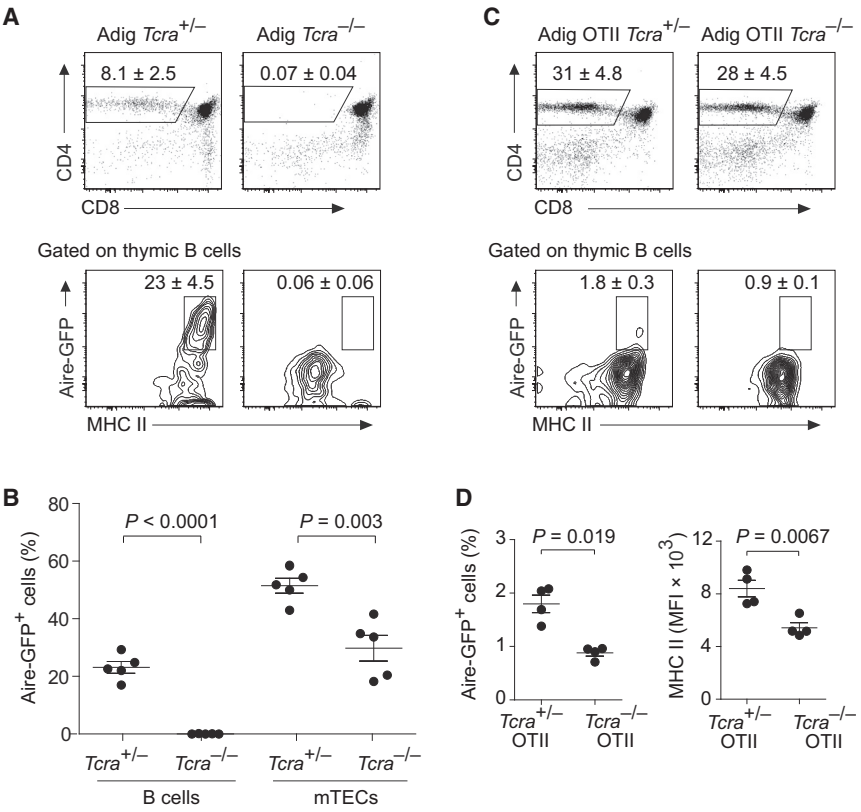

Figure 4. A CD4 SP Compartment with Diverse TCRs Is Required for Thymic B Cell Licensing

(A) Thymic CD4 versus CD8 profiles in Adig *Tcra*$^{+/-}$ and Adig *Tcra*$^{-/-}$ mice and MHCII versus Aire-GFP in gated CD19^{+} thymic B cells. The MFI ± SEM of MHCII was 37,000 ± 2,800 on Adig *Tcra*$^{+/-}$ (n = 5) and 5,700 ± 1,200 on Adig *Tcra*$^{-/-}$ thymic B cells (n = 6) ($p < 0.0001$).

(B) Frequency of Aire-GFP^{+} cells among thymic B cells or mTECs from Adig *Tcra*$^{+/-}$ (n = 5) and Adig *Tcra*$^{-/-}$ (n = 6) mice.

(C) Thymic CD4 versus CD8 profiles in Adig OTII *Tcra*$^{+/-}$ or Adig OTII *Tcra*$^{-/-}$ mice and MHCII versus Aire-GFP on thymic B cells.

(D) MHCII MFI on thymic B cells from Adig OTII *Tcra*$^{+/-}$ and Adig OTII *Tcra*$^{-/-}$ mice (left). Frequency of Aire-GFP^{+} cells among thymic B cells (right).

n = 4 (C and D each). See also Figure S4.

mechanisms. We therefore directly assessed the reactivity of CD4 SP thymocytes selected in the absence or presence of thymic B cells. We reasoned that the tolerogenic potential of licensed thymic B cells might in particular comprise activated B cell autoantigens, so that T cells selected in the absence of thymic B cells should be hyper-responsive to activated B cells. Indeed, upon co-culture with anti-CD40-activated syngeneic B cells, CD4 SP thymocytes from B-cell-deficient mice proliferated substantially more than controls from WT mice, whereas their response to allogenic B cells was indistinguishable (Figure 7C). Together, these findings supported the idea that in the absence of thymic B cells, the CD4^{+} T cell repertoire is incompletely tolerized.

In order to directly assess whether and how an antigen that is expressed in thymic B cells in a licensing-dependent manner is presented and contributes to CD4^{+} T cell tolerance, we employed the Aire-HCO transgenic mouse model. In addition to the h*CD2* Aire-reporter, Aire-HCO mice also express a chimeric influenza hemagglutinin (HA) protein from a bi-cistronic mRNA. The expression of this neo-self antigen in mTECs and thymic B cells from Aire-HCO mice faithfully recapitulated the expression pattern of endogenous Aire mRNA (Figure 7D, compare 1E). Within the B cell lineage, the selective expression of the Aire-HCO transgene exclusively in licensed thymic B cells but not resting peripheral B cells thereby not only mimicked the licensing-dependent upregulation of Aire-dependent TRAs, but also emulated Aire-independent changes in the transcriptional profile of B cells associated with the acquisition of an activated phenotype. To address how this expression pattern correlated with antigen presentation, purified thymic B cells, mTECs, thymic DCs, and peripheral B cells from Aire-HCO mice were cultured together with the HA-specific CD4^{+} T cell hybridoma A5 (Aschenbrenner et al., 2007). mTECs elicited the strongest response, consistent with their pronounced capacity to present endogenously expressed antigens on MHCII (Figure 7E; Aschenbrenner et al., 2007; Hinterberger et al., 2010). Remarkably, despite the differential Aire-HCO mRNA expression in thymic B cells but not DCs, both cell types presented the HA epitope with a similar efficacy (Figure 7E), whereas no presentation was detectable with peripheral B cells (Figure S6). In Aire-HCO → WT BM chimeras, presentation of HA on thymic B cells remained intact, whereas presentation by DCs was abolished, indicating that HA presentation by DCs from unmanipulated Aire-HCO thymi reflected the acquisition of mTEC-derived antigen (Figure 7F). Thus, thymic B cells directly present an endogenously expressed antigen whose induction reflects and depends upon intrathymic B cell licensing.

B Cell Presentation of an Endogenous, Licensing-Dependent Antigen Induces Negative Selection

Expectedly, HA-specific CD4 SP cells underwent negative selection in Aire-HCO TCR-HA double-transgenic mice, in which both mTECs and thymic B cells express the neo-antigen (Figure S7A). Negative selection likewise occurred in TCR-HA Aire-HCO → WT BM chimeras, indicating that hematopoietically expressed HA was sufficient for clonal deletion (Figure S7B). Deletion through HA expression in BM-derived cells also remained intact in TCR-HA Aire-HCO ΔDC → WT chimeras, which lack CD11c^{+} cells due to expression of diphteria toxin in CD11c^{+} cells (Figures S7C and 7D; Ohnmacht et al., 2009). This demonstrated that DCs were not required as indirect presenters of exogenously acquired antigen and also rendered the small population of CD11c^{+}Aire-reporter^{+} non-B cells (see Figure 1B) an unlikely essential source of negatively selecting antigen.

Together with our analyses of antigen presentation (Figures 7E and 7F), these findings strongly supported the idea that direct presentation of an endogenous, licensing-dependent antigen by thymic B cells was sufficient for negative selection.

Finally, we established a reductionist model to assess the capacity of immigrating peripheral B cells to mediate negative selection. In exploratory experiments, the transfer of peripheral Aire-HCO B cells into TCR-HA recipients failed to measurably affect the thymic development of TCR-HA$^+$ CD4 SP cells. We reasoned that this was due to the limited thymic B cell chimerism achieved in this way (typically less than 0.1%; see Figure 2C), possibly owing to competition at the level of thymic entry and/or available niches. Hence we conditioned CD45.1 recipient mice by irradiation and reconstituted them with mixed CD45.1 WT and CD45.2 TCR-HA *Rag2*$^{-/-}$ BM cells prior to adoptively transferring splenic CD45.1$^+$CD45.2$^+$ B cells from Aire-HCO or WT donors (Figure 7G). 3 weeks after transfer, CD45.1$^+$CD45.2$^+$ donor B cells that had immigrated into the thymus still accounted for 36% ± 7.6% of thymic B cells. At this point in time, the transit of donor BM-derived thymocytes through the DN, DP, and SP compartments had reached a steady state, with 22% ± 3.4% of the progeny of TCR-HA *Rag2*$^{-/-}$ BM cells residing in the CD4 SP compartment in recipients of WT B cells (i.e., in the absence of cognate antigen). By contrast, recipients of Aire-HCO B cells displayed a significantly reduced frequency of CD4 SP cells among TCR-HA *Rag2*$^{-/-}$ thymocytes but not among polyclonal bystander cells (Figures 7H and 7I). Thus, a self-antigen that is exclusively expressed by immigrating peripheral B cells in a licensing-dependent manner mediates central tolerance.

DISCUSSION

Our findings reveal an intricate interplay between B cells and CD4 SP thymocytes that endows thymic B cells with potent tolerogenic features. Mature peripheral B cells readily acquired a licensed phenotype when exposed to the thymic microenvironment, indicating that intrathymic B lymphopoiesis (Akashi et al., 2000) is not a crucial prerequisite for the unique phenotype of thymic B cells. Moreover, the paucity of B cell precursors in the thymus suggests that most thymic B cells might stem from immigration. However, our data certainly do not dismiss the notion that an as yet unknown fraction of thymic B cells arises through an intrathymic developmental pathway (Perera et al., 2013). Importantly, irrespective of the exact quantitative contribution of intrathymic and conventional B lymphopoiesis, the homogeneity among thymic B cells regarding MHCII and CD80 expression indicates that licensing applies to essentially all cells regardless of their origin. Conceivably, Aire expression might lag behind MHCII and CD80 up-regulation because it might require cell division-associated permissive chromatin alterations.

Cross-talk-dependent induction of MHCII and CD80 and postmitotic onset of Aire expression also characterize mTEC maturation (Akiyama et al., 2008; Gray et al., 2007; Hikosaka et al., 2008; Rossi et al., 2007). However, whereas B cell licensing requires CD40 signals in the context of cognate CD4 SP cell-B cell interactions, this signaling axis is of lesser relevance in mTEC maturation. Although CD40 signals from autoreactive CD4 SP cells are thought to sustain mTEC homeostasis (Irla et al., 2008; White et al., 2008), the acquisition of an MHCII^{hi}CD80$^+$Aire$^+$ mTEC phenotype as such is orchestrated by Rank signals. Unlike CD40 signals for B cell licensing, Rank signals for mTEC maturation are supplied not only by CD4 SP cells, but also by LTi cells, invariant γδ T cells, and iNKT cells (Akiyama et al., 2008; Hikosaka et al., 2008; Roberts et al., 2012; Rossi et al., 2007; White et al., 2014). This difference might explain the ontogenetically early appearance of mature mTECs, whereas the licensed phenotype in thymic B cells coincides with the perinatal emergence of SP thymocytes (our unpublished observation).

The cognate T cell-B cell interplay that initiates thymic B cell licensing bears striking resemblance to the GC reaction. However, whereas T cell-B cell interactions in GCs are driven by BCR-mediated antigen internalization and MHCII-restricted presentation of foreign antigens (Victora and Nussenzweig, 2012), the corresponding signal exchange in the thymus appears to center around the BCR-independent display of self antigens. The exact nature of the licensing-inducing self-antigens that are presented by immigrating B cells remains to be determined. Conceivably, the relevant peptide-MHCII (pMHC) ligands that are recognized by an as yet unknown fraction of polyclonal CD4 SP thymocytes are derived from a heterogeneous group of endogenously expressed proteins. Such endogenous self-antigens constitute a considerable part of the resting B cell pMHCII-ligandome (Brooks et al., 1991; Dengjel et al., 2005), and the nascent CD4 SP compartment is apparently not (yet) fully purged of the respective autoreactive specificities.

Another parallel between the GC reaction and thymic B cell licensing is the essential role of CD40 signaling (Victora and Nussenzweig, 2012). Strikingly, cross-linking of the BCR simultaneous to CD40 stimulation, that is, mimicking a key aspect of GC formation (Yuseff et al., 2013), selectively suppressed Aire induction but not MHCII upregulation. These findings offer an explanation for why GC B cells do not express Aire, although seemingly identical intercellular interactions elicit proliferation, class-switching, and enhanced APC features in both GC B cells and thymic B cells.

In addition to providing thymic B cells with enhanced APC features such as elevated MHCII and CD80, CD40 signaling was shown to also control the size of the thymic B cell pool, which in sum should quantitatively enhance the tolerogenic potential of the thymic B cell population as a whole (Fujihara et al., 2014). Importantly, our findings strongly suggest that besides these quantitative effects, intrathymic licensing is likely to have a substantial qualitative influence on the self antigen spectrum displayed by thymic B cells.

First, the licensing-dependent pMHCII ligandome should reflect Aire-mediated changes in the transcriptome of thymic B cells. Remarkably few Aire-induced transcripts in thymic B cells are Aire-dependently upregulated in mTECs or eTACs (Derbinski et al., 2005; Gardner et al., 2008), supporting the notion that Aire's function as a transcriptional regulator is cell context dependent. Importantly, there is accruing evidence that Aire also orchestrates APC functions beyond promiscuous gene expression, for instance by regulating chemokine expression (Laan et al., 2009; Lei et al., 2011), cellular differentiation, or antigen presentation as such (Anderson et al., 2005; Nishikawa et al., 2010; Yano et al., 2008). All these possibilities need to be considered in the context of Aire's potential role(s) in thymic B cells.

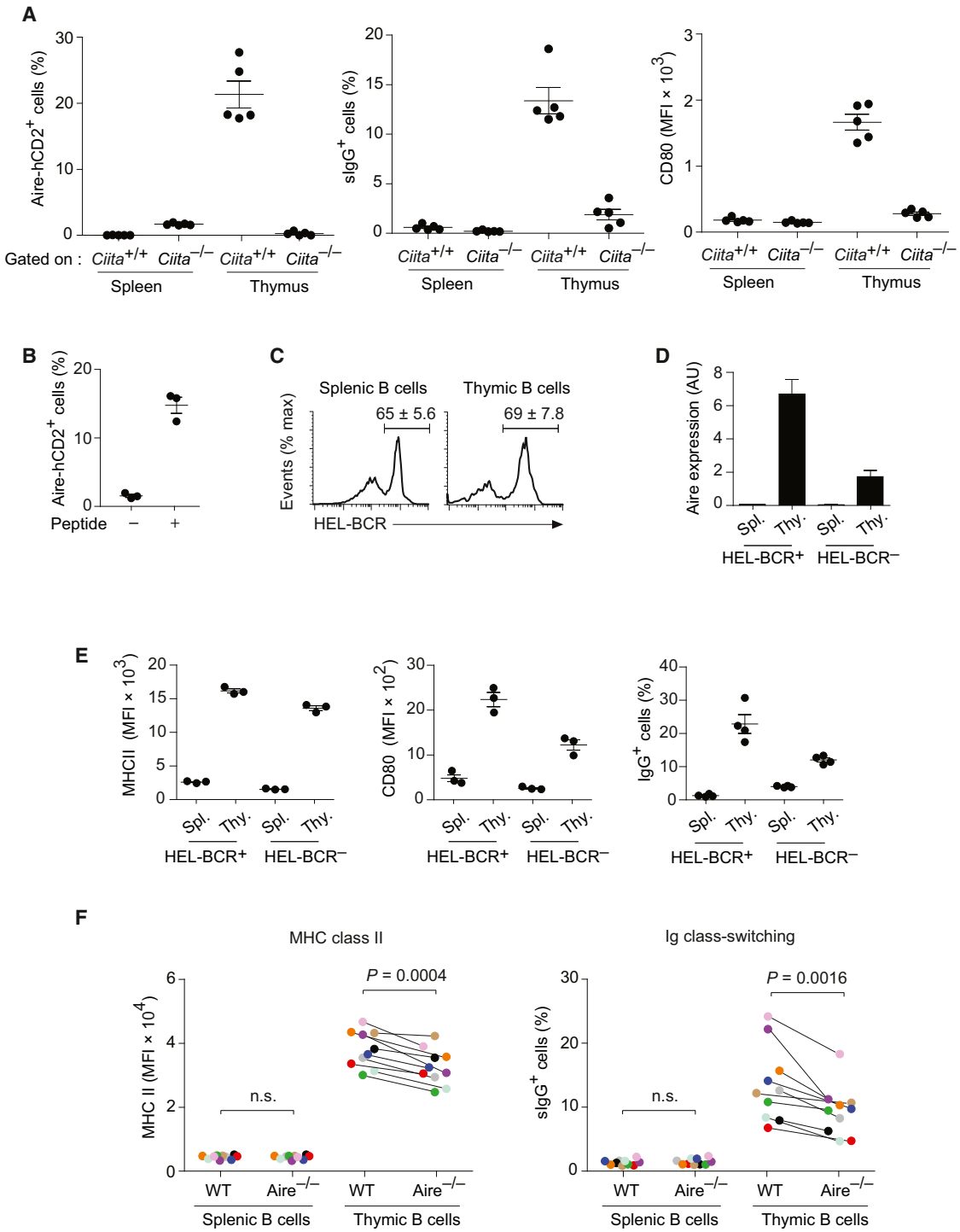

Figure 5. BCR-Specificity-Independent MHCII-Restricted Presentation of Self to Autoreactive CD4 SP Cells Initiates B Cell Licensing

(A) Expression of the Aire-hCD2 reporter, surface IgG, and CD80 on MHCII-sufficient (gated CD19$^+$CD45.2$^-$CD45.1$^+$ cells) or MHCII-deficient (gated CD19$^+$ CD45.2$^+$CD45.1$^-$ cells) splenic and thymic B cells in mixed BM chimeras generated by reconstitution of lethally irradiated CD45.1$^+$ mice with a 1:1 mixture of BM from Aire-HCO *Ciita*$^{+/+}$ CD45.1$^+$ and Aire-HCO *Ciita*$^{-/-}$ CD45.2$^+$ mice.

(B) Expression of Aire-hCD2 on Aire-HCO splenic B cells after 3 day co-culture with DO11.10 *Rag2*$^{-/-}$ CD4 SP cells with or without OVA peptide (n = 3).

(C) Percentage ± SEM of HEL-BCR-expressing cells among splenic and thymic B cells from SW_{HEL} mice (n = 4).

(D) Aire mRNA in HEL-BCR$^+$ and HEL-BCR$^-$ B cells from spleen or thymus of SW_{HEL} mice. Data show mean values ± SEM of three replicates.

(E) MFI of MHCII, CD80, and surface IgG on HEL-BCR$^+$ or HEL-BCR$^-$ B cells from spleen or thymus of SW_{HEL} mice (n = 3 or 4).

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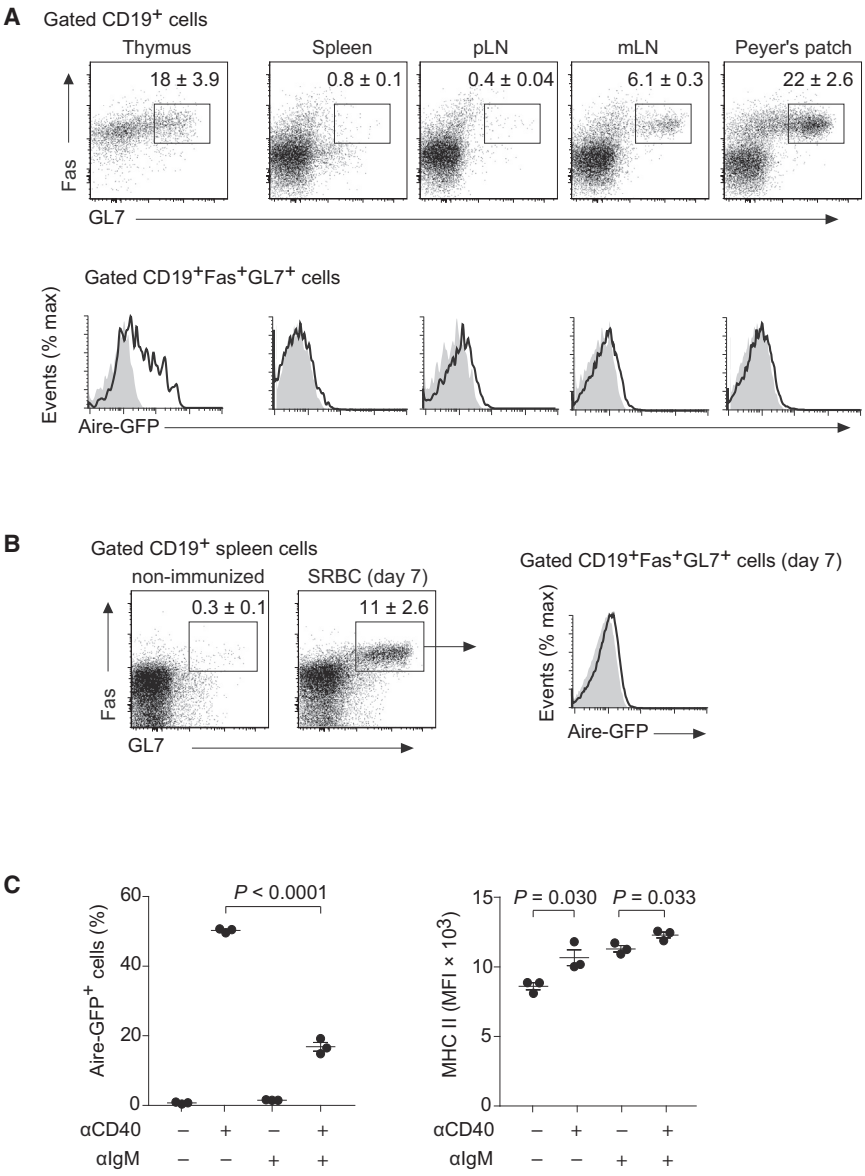

Figure 6. BCR Stimulation Counteracts CD40-Mediated Aire Induction

(A) Steady-state Fas$^+$GL7$^+$ B cells in the thymus, spleen, popliteal (p)LN, mesenteric (m)LN, and Peyer's patches of Adig mice (n = 3). Histograms show the expression of Aire-GFP in gated CD19$^+$Fas$^+$GL7$^+$ cells from Adig mice compared to WT controls (gray histograms).

(B) Germinal centers were induced in Adig or WT mice through SRBC immunization (n = 4 each). On day 7, CD19$^+$ splenocytes were analyzed for surface expression of Fas and GL7. The histogram overlay shows the expression of Aire-GFP in CD19$^+$Fas$^+$GL7$^+$ cells from Adig mice (open histograms) or WT controls (gray histograms).

(C) Percentage of Aire-GFP$^+$ cells and MHCII MFI in Adig spleen B cells that had been cultured for 3 days with or without agonistic anti-CD40 antibody (10 μg/ml) and anti-IgM antibody (20 μg/ml) (n = 3). Representative of two independent experiments.

(F) Lethally irradiated mice (CD45.1$^+$) were reconstituted with a 1:1 mixture of congenically marked BM cells from WT (CD45.1$^+$) and *Aire*$^{-/-}$ (CD45.2$^+$) mice. 5 weeks later, donor B cells in spleen and thymus were analyzed for expression of MHCII (left) and sIgG (right) (n = 10). Experimental pairs from individual mice are color coded. Statistical significance was assessed via a paired Student's t test.

See also Figure S5.

B cells, because self-reactive B cells are thereby deprived of cognate help (Goodnow et al., 2010). This requires not only stringent helper cell tolerance toward exogenously derived self-determinants presented by GC B cells as a consequence of antigen capture via their hypermutated BCR, but also that the T helper cell repertoire is efficiently purged of reactivity toward any endogenously derived B cell autoantigen that is concurrently presented. We propose that thymic B cell licensing is crucial to pre-empt T cell recognition of activated B cell autoantigens in an inflammatory context in secondary lymphoid tissues. Future work combining tissue-specific depletion of B cells selectively in the thymus, conditional ablation of Aire in B cells, and/or in-depth TCR repertoire analyses (Perry et al., 2014) under such conditions can be expected to shed more light on how thymic B cell licensing shapes the T cell repertoire.

Second, and regardless of Aire's contribution, the spectrum of licensing-dependent autoantigens expressed by thymic B cells is expected to comprise a variety of B-cell-specific self antigens that are otherwise selectively expressed or upregulated in activated peripheral B cells. Hence, the pMHCII ligandome displayed by thymic B cells for central T cell tolerance might to a substantial degree emulate the composition of MHCII-bound peptides on GC B cells, including B lineage-specific neo-epitopes generated through isotype class switching or possibly also somatic hypermutation.

Robust T helper cell tolerance is deemed key to the control of BCR-hypermutation-related neo-autoreactivity among GC

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are described in the Supplemental Information section.

Mice

References for mouse strains can be found in Supplemental Experimental Procedures. All animal studies were approved by local authorities (Regierung von Oberbayern).

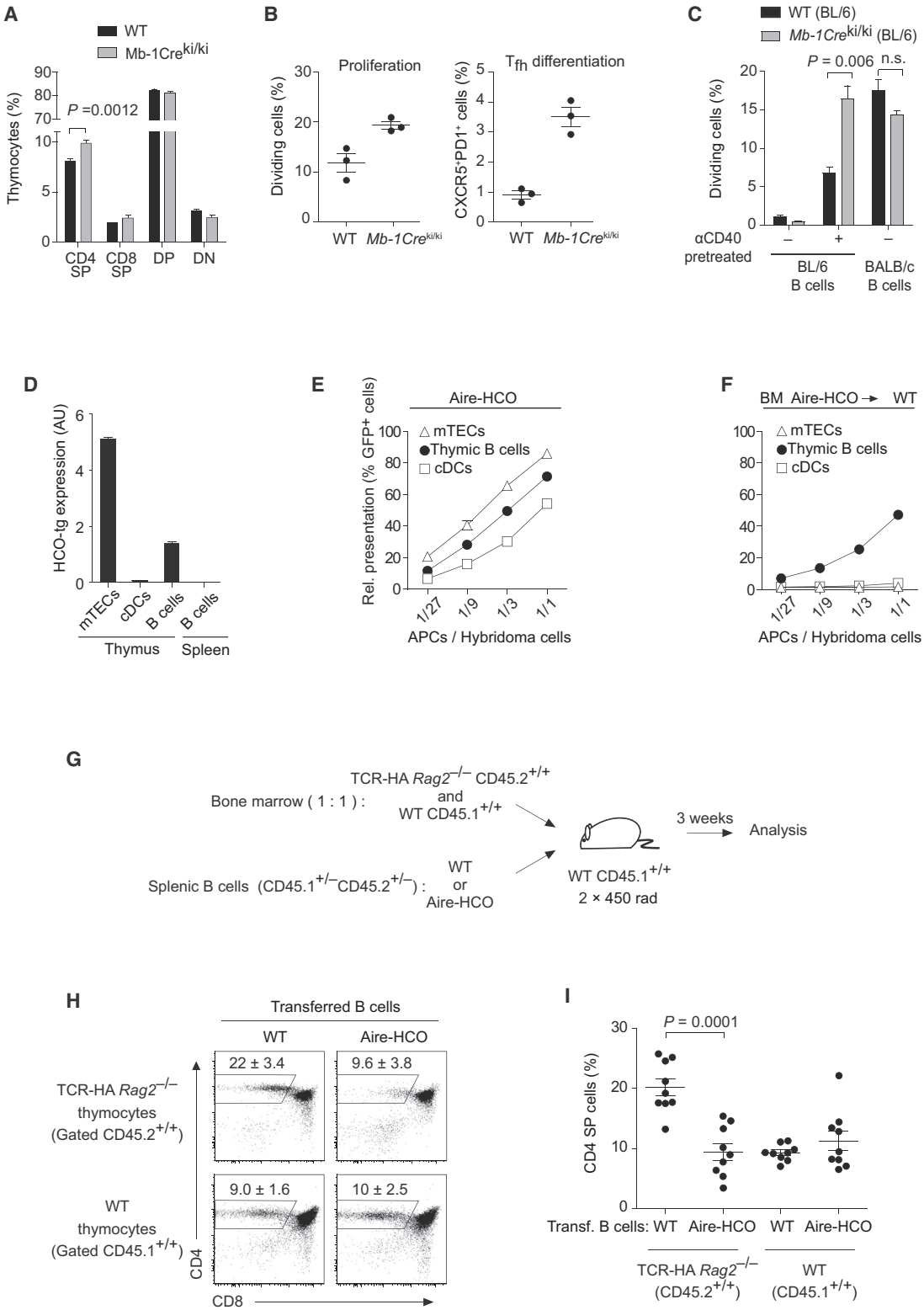

Figure 7. Direct Presentation of a Licensing-Associated Neo-antigen by Thymic B Cells Promotes Negative Selection

(A) Frequency of thymocyte subsets in WT and B-cell-deficient (*Mb1*-Cre$^{ki/ki}$) mice (n = 4).

(B) Purified peripheral CD4$^+$ T cells (CD45.1) from WT or *Mb1*-Cre$^{ki/ki}$ mice were CFSE labeled and intravenously injected into CD45.2 WT recipients. Seven days after transfer, the frequency of dividing donor T cells that had diluted the CFSE label was determined. Spontaneous Tfh cell differentiation was assessed by determining the frequency of CXCR5$^+$PD-1$^+$ cells among donor cells that had undergone at least one division (n = 3 each).

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Antibodies and Flow Cytometry

Stainings were performed according to standard procedures. For intracellular Aire staining, fixed and permeabilized cells were stained with Alexa Fluor 660-conjugated mAb 5H12-2 (eBioscience).

Imaging Flow Cytometry

Cells were stained for surface markers, fixed, permeabilized, and intracellularly stained with an Alexa Fluor 660-conjugated anti-Aire mAb. DAPI was added immediately prior to the analysis. Images were acquired with the ImageStream imaging flow cytometer (Amnis), and data were analyzed with Ideas 6.0 software.

In Vitro Stimulation of B Cells

Purified B cells (2×10^5) were cultured for 3 days with or without 2×10^4 irradiated (3,000 rad) ST2 or ST2-RankL (Nutt et al., 1999) BM stroma cells in flat-bottom 96-well plates. Where indicated, agonistic anti-CD40 mAb (FGK45, Bio X Cell) (10 μg/ml), agonistic anti-Rank mAb (AF692, R&D Systems), and/or anti IgM F(ab')2 (Jackson Immuno Research) (20 μg/ml) were added.

Sheep Red Blood Cell Immunization

Mice were immunized i.v. with 4×10^8 sheep red blood cells (SRBCs) in 200 μl PBS. After 7 days, spleen cells were analyzed by flow cytometry.

Adoptive B Cell and T Cell Transfer

B cells (1×10^7) were i.v. injected in 200 μl PBS into the lateral tail vein of recipient mice. Where indicated, cells were labeled with 1 μM of CellTrace Violet (Life Technologies). After 7 days, organs were collected and single-cell suspensions were analyzed by flow cytometry.

Purified LN T cells (1×10^7) were labeled with CFSE (Life Technologies) and i.v. injected into the lateral tail vein of congenic recipient mice. After 7 days, spleen cells were analyzed by flow cytometry.

In Vitro Stimulation of CD4 SP Cells with B Cells

CD4 SP cells were enriched by depletion of total thymocytes with biotin conjugated anti-CD8, anti-CD25, and anti-SAV MicroBeads (Miltenyi Biotech). 2×10^5 CFSE-labeled cells were co-cultured with 2×10^5 syngeneic or allogeneic B cells. Where indicated, B cells had been anti-CD40 activated before (10 μg/ml). After 3 days, cells were harvested and analyzed.

Intrathymic Injection

FACS sorted $CD19^+IgG^-$ LN B cells (5×10^5) were injected in 3 μl PBS into one thymic lobe. After 7 days, the thymus was collected and analyzed by flow cytometry.

Antigen-Presentation Assay

2×10^4 A5 hybridoma cells were cultured with 2×10^4 APCs in 200 μl IMDM medium supplemented with 1% FCS. After 17 hr, cells were harvested and GFP expression was measured by flow cytometry (Aschenbrenner et al., 2007).

Preparation of Thymic APCs

Thymic epithelial cells and DCs were isolated as described elsewhere (Aschenbrenner et al., 2007). Surface markers for sorting were as follows: mTECs, $CD45^-EpCAM^+Ly51^-$; B cells, $CD45^+CD19^+$; pDCs, $CD45^+CD11c^{int}CD45RA^+$; $Sirp\alpha^+$ cDCs, $CD45^+CD11c^{hi}CD172a^+$; $Sirp\alpha^-$ cDCs, $CD45^+CD11c^{hi}CD172a^-$.

Bone Marrow Chimeras

BM was B and T cell depleted using biotinylated B220, CD8, and CD4 mAbs and streptavidin beads (Miltenyi Biotech). Recipient mice were irradiated with 2×450 rad and reconstituted with 1×10^7 bone marrow cells. For the generation of mixed BM chimeras, BM cells from the indicated donors (5×10^6 each) were mixed at a ratio of 1:1.

Statistical Analysis

Unless indicated otherwise, statistical significance was assessed using the two-tailed Student's t test with unequal variance.

ACCESSION NUMBERS

The data presented in this paper have been deposited to the European Nucleotide Archive under accession number ENA: PRJEB9511.

SUPPLEMENTAL INFORMATION

Supplemental Information include seven figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.05.013.

AUTHOR CONTRIBUTIONS

T.Y. carried out or contributed to all experiments; J.N., M.H., and M.S. contributed to animal experiments; S.K., S.P., B.K., and B.B. performed analyses of RNA-seq data; N.G., E.L., and N.I. provided mice and reagents; T.Y. analyzed Pax5-reporter mice with M.B.; T.Y., J.N., and L.K. designed experimental strategies; and T.Y., B.K., and L.K. wrote the manuscript.

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(C) Purified CD4 SP thymocytes from WT or *Mb1*-$Cre^{ki/ki}$ mice were CFSE labeled and co-cultured with syngenic (BL/6) or allogenic (BALB/c) peripheral B cells. Where indicated, B cells were pre-activated with agonistic CD40 antibody. After 3 days of co-culture, the frequency of CD4 SP T cells that had diluted the CFSE label was determined (n = 3).
(D) Aire-HCO mRNA in mTECs, thymic cDCs, thymic B cells, and splenic B cells from Aire-HCO mice (mean ± SEM of three replicates).
(E) Presentation of HA by mTECs, thymic cDCs, or thymic B cells from Aire-HCO mice (representative of two experiments with pooled cells from $n \geq 3$).
(F) Presentation of HA by mTECs, thymic cDCs, or thymic B cells from Aire-HCO → WT BM chimeras (representative of two experiments with pooled cells from $n \geq 3$).
(G) Experimental strategy to assess the consequences of licensing-associated antigen expression confined to immigrating B cells.
(H) Thymocyte composition in WT mice 3 weeks after irradiation and reconstitution with mixed BM (TCR-HA *Rag2*$^{-/-}$ and WT; 1:1) and adoptive transfer of peripheral B cells from Aire-HCO or WT mice (control).
(I) Summary of the percentage of HA-specific or bystander CD4 SP cells (n = 9 each).
See also Figures S6 and S7.

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