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J Immunol 2017; 198:3058-3068; Prepublished online 8 March 2017;
doi: 10.4049/jimmunol.1601285
http://www.jimmunol.org/content/198/8/3058

Supplementary Material
http://www.jimmunol.org/content/suppl/2017/03/08/jimmunol.1601285.DCSupplemental

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Distinct and Overlapping Functions of TEC Kinase and BTK in B Cell Receptor Signaling

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The Tec tyrosine kinase is expressed in many cell types, including hematopoietic cells, and is a member of the Tec kinase family that also includes Btk. Although the role of Btk in B cells has been extensively studied, the role of Tec kinase in B cells remains largely unclear. It was previously shown that Tec kinase has the ability to partly compensate for loss of Btk activity in B cell differentiation, although the underlying mechanism is unknown. In this study, we confirm that Tec kinase is not essential for normal B cell development when Btk is present, but we also found that Tec-deficient mature B cells showed increased activation, proliferation, and survival upon BCR stimulation, even in the presence of Btk. Whereas Tec deficiency did not affect phosphorylation of phospholipase Cγ or Ca2+ influx, it was associated with significantly increased activation of the intracellular Akt/S6 kinase signaling pathway upon BCR and CD40 stimulation. The increased S6 kinase phosphorylation in Tec-deficient B cells was dependent on Btk kinase activity, as ibrutinib treatment restored pS6 to wild-type levels, although Btk protein and phosphorylation levels were comparable to controls. In Tec-deficient mice in vivo, B cell responses to model Ags and humoral immunity upon influenza infection were enhanced. Moreover, aged mice lacking Tec kinase developed a mild autoimmune phenotype. Taken together, these data indicate that in mature B cells, Tec and Btk may compete for activation of the Akt signaling pathway, whereby the activating capacity of Btk is limited by the presence of Tec kinase. The Journal of Immunology, 2017, 198: 3058–3068.

1R.W.H. and O.B.J.C contributed equally to this work.
2Received for publication July 25, 2016. Accepted for publication February 15, 2017.
3This work was supported in part by Dutch Arthritis Foundation (Reumafonds) Grants 09-1-302 and 13-2-301 and by Dutch Cancer Society Grant 2014-6564.
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5The online version of this article contains supplemental material.
6Abbreviations used in this article: BM, bone marrow; CLL, chronic lymphatic leukemia; GC, germinal center; PC, plasma cell; PLC, phospholipase C; Tfh, follicular Th; WT, wild-type.
7Copyright © 2017 by The American Association of Immunologists, Inc. 0022-1767/17/$30.00
8www.jimmunol.org/cgi/doi/10.4049/jimmunol.1601285
further investigate the role of Tec in mature B cells and during immune responses.

We show in this study that in Tec-deficient mice in vitro B cell activation following BCR stimulation was increased. This was associated with enhanced activation of the Akt/S6 kinase pathway upon BCR or CD40 engagement in Tec-deficient B cells, which was dependent on Btk kinase activity. Moreover, Tec-deficient mice manifest increased in vivo B cell responses and a mild autoimmunity phenotype. Taken together, these data indicate that Btk activity may be limited by competition with Tec kinase.

Materials and Methods

Mice

Btk-deficient (4) and Tec-deficient (5) mice were backcrossed for >10 generations onto a C57BL/6 background. Mice were analyzed between 8 and 14 or 34 wk of age for autoimmunity studies. Mice were bred and kept in the experimental animal facility of the Erasmus Medical Center under specific pathogen-free conditions. All experiments were approved by the Erasmus University Medical Center Animal Ethics Committee.

MACS purification and B cell cultures

Bone marrow (BM) cells were cultured with 150 U/ml mouse rIL-7 (R&D Systems) for 5 d, followed by 2 d without rIL-7.

Naïve B cells from spleens were MACS purified and cultured in vitro for 1–4 d as described (10). Cells were stimulated with (combinations of) 10 μg/ml F(ab′)2 anti-mouse IgM fragments (Jackson ImmunoResearch Laboratories), 5 ng/ml LPS (own production) for cell cycle analysis and various concentrations (Sigma-Aldrich) for plasmablast cultures, 5 ng/ml PMA (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich), 0.1 μg/ml mouse rIL-4 (PeproTech), or anti-CD40 10 μg/ml (clone 3/23; BD Biosciences).

Flow cytometry, cell cycle analysis, CSFE labeling, and measurement of Ca2+ mobilization

Flow cytometry was performed using Abs and reagents as described (11). For phosphoflow, total spleen cells were stained for extracellular markers and stimulated with (combinations of) anti-IgM F(ab′)2 fragments (4 or 25 μg/ml), mouse rIL-4 (0.1 μg/ml), and anti-CD40 (4 or 20 μg/ml) at 37°C. Cells were then fixed with Cytofix and permeabilized with Phosflow perm buffer III (BD Biosciences) and stained for anti-pS6 ribosomal protein (S240/244; Cell Signaling Technology), anti-pAkt (S473; BD Biosciences), and measured on an ABI 7300 (Applied Biosystems) for 5 d, followed by 2 d without rIL-7.

Bone marrow (BM) cells were cultured with 150 U/ml mouse rIL-7 (R&D Systems) and analyzed using FlowJo software (Tree Star).

Quantitative RT-PCR

RNA was isolated using the GenElute RNA isolation kit (Sigma-Aldrich) and cDNA was made using the ReverTra A minus first-strand cDNA synthesis kit (Thermo Fisher Scientific). Germline transcripts of Igα and Igα loci were determined using the following primers: kα0.0.8 and kα0.1.1 (14), and λα0.0.1, λα0.0.2, and λα0.0.3 (15).

To detect human TEC transcripts, we used forward primer 5′-ACATGCTTTTTGCTTACCTC-3′ and reverse primer 5′-GGTACCGAACGCTCTGCTGTGTA-3′; for human BTK transcripts we used forward primer 5′-TGGCTGAAACAGTGGTTCCTGA-3′ and reverse primer 5′-TGCTCCATTTCACGTAGCTTCTTACCT-3′. Expression was normalized to ubiquitin C expression (forward primer 5′-ATTTGTTGGTCGGGCGTCT-3′ and reverse primer 5′-TGCTTCATTACTCGATTTTGC-3′).

Quantitative PCR was performed using SYBR Green master mix (Thermo Fisher Scientific) and measured on an ABI 7300 (Applied Biosystems) machine using 7300 software (Applied Biosystems).

DNP-Ficoll and TNP–KLH immunization

For T cell–independent immunization, 50 μg of DNP(49–55)Ficoll (Biosearch Technologies) in PBS was injected i.p. Serum was collected at baseline and 2, 4, and 7 d after immunization. For T cell–dependent immunization, 100 μg of TNP29–keyhole limpet hemocyanin (KLH; Biosearch Technologies) and 0.8 mg of Imject aluminum hydroxide adjuvant (Thermo Fisher Scientific) in PBS was injected i.p. in mice at day 0, followed by a boost immunization with 100 μg of TNP29–KLH in PBS 35 d later. Mice were sacrificed 7 d after the boost.

Ibrutinib treatment

Mice were treated in vivo with ibrutinib as described (10) and sacrificed 3 h later for analysis. For in vitro studies, 1 μM ibrutinib was added to the culture medium.

Influenza virus infection

X-31 influenza virus particles (103) diluted in PBS were given intranasally (16). Anti-hemagglutinin inhibition assay was performed on serum collected 15 and 25 d postinfection (17).

Immunohistochemistry

Cryostat sections (6 μm) were cut from OCT compound–embedded tissues, fixed in acetone, and processed as described (18).

Ig subclass concentrations and autoreactivity

Isotype-specific serum Ab levels (19) and anti-DNA IgM and IgG autoantibody levels (11) were measured as described. Ig autoreactivity was analyzed by HEp2-staining as described (10).

Results

Development of Tec-deficient B cells is comparable to WT B cells

To confirm that B cell development in the BM of Tec-deficient mice is normal, as previously described (5), we analyzed BM cells of WT, Tec-deficient (Tec−/−), Btk-deficient (Btk−/−), and Tec/Btk double-deficient (Tec/Btk−/−) mice on the C57BL/6 background by flow cytometry.

In the BM, total cell numbers were comparable in all mouse groups, but total B-lineage cell numbers were lower in Tec/Btk−/− mice (Supplemental Fig. 1A, 1B). Similar to findings on a mixed background (5), Tec/Btk−/− mice showed a developmental block at the large pre–B cell stage (Supplemental Fig. 1C). Btk−/− B cells, but not Tec−/− B cells, showed a partial developmental block at the large pre–B cell stage (as described previously in Ref. 12). This indicates that lack of Tec could be completely compensated by Btk.

In Tec−/− mice, pre-BCR function was unaffected, as analyzed by modulation of cell surface markers, germline transcription over unarranged Ig L chain loci, and developmental progression of pre–B cells in vitro (Supplemental Fig. 1D, 1F), indicating that lack of Tec could be completely compensated by Btk.

Finally, because we found that Btk cooperates with the linker protein Slp65 as a tumor suppressor at the pre–B cell stage, we analyzed the effects of Tec expression on pre–B cell tumor formation. Btk−/− mice do not develop pre–B cell tumors, but the incidence of pre–B cell lymphomas is higher in Slp65/Btk−/− mice than in Slp65−/− mice (20). However, we did not observe tumors in Tec/Btk−/− mice or differences in pre–B cell tumor frequencies between Tec/Btk/Slp65−/− mice and Slp65/Btk−/− mice (data not shown). Therefore, Tec does not have a tumor suppression function at the pre–B cell stage that may be masked by the expression of Btk.

Taken together, these data suggest that although Tec and Btk have complementary roles in pre-BCR–mediated developmental progression of pre–B cells (5), B cell development is normal in Tec−/− mice.

Survival, proliferation, and activation are enhanced in mature Tec−/− B cells in vitro

Total splenocyte and B cell numbers were lower in Btk−/− and Tec/Btk−/− mice, compared with WT and Tec−/− mice (Supplemental
Fig. 1A, 1B). Moreover, we found a relative increase in CD21+/CD23lo marginal zone B cells and, as previously shown (5), a decrease in mature IgD+IgM+ B cells in the spleens of Tec/Btk−/− mice (Supplemental Fig. 1G and data not shown). Nevertheless, in these analyses we did not detect differences in the B cell compartments in the spleen or peritoneal cavity between Tec−/− and WT mice (Supplemental Fig. 1G and data not shown).

Next, we compared survival, proliferation, and activation of mature B cells in WT and Tec−/− mice. MACS-purified naive splenic B cells were cultured with either LPS (5 ng/ml), a combination of PMA (5 ng/ml) and ionomycin (1 μg/ml), anti-IgM F(ab′)2 fragments (10 μg/ml) with or without rIL-4 (0.1 μg/ml), or anti-CD40 (10 μg/ml) with or without rIL-4. After 2 d of culture, propidium iodide staining revealed that unstimulated Tec−/− cultures had significantly more proliferating cells than did unstimulated WT cultures (Fig. 1A). Additionally, following anti-IgM stimulation, Tec−/− B cells had increased proportions of proliferating cells (∼43%, compared with ∼26% in WT B cells) and decreased proportions of apoptotic cells (21%, compared with ∼38% in WT B cells) (Fig. 1A). Addition of IL-4 to the cultures decreased apoptosis in WT, but not in Tec−/− B cell cultures, suggesting that the beneficial effect of Tec deficiency on cell survival is mediated through similar downstream pathways, possibly the Akt pathway. The other stimulations showed no significant differences. CFSE labeling confirmed that there were more cell divisions upon anti-IgM stimulation, with or without rIL-4 (Fig. 1B). Anti-CD40–stimulated Tec−/− B cells showed slightly increased cell division in Tec-deficient B cells, but the other stimulation conditions showed no differences (Fig. 1B and data not shown).

We further analyzed the B cell activation status by flow cytometry after 1 and 3 d of activation in vitro. After 3 d in culture, expression of activation markers CD25 and CD69 was increased with anti-CD40 and rIL-4, as was the cell size when measured by forward scatter, showing an enhanced activation status of the Tec−/− B cells (Fig. 1C). Similar but less pronounced increases were found at day 1 (data not shown).

In summary, Tec-deficient mature B cells show increased activation, proliferation, and survival upon in vitro BCR stimulation with anti-IgM or anti-CD40, particularly in combination with rIL-4, compared with WT B cells.

**BCR-dependent PLCγ activation is not affected in Tec−/− B cells**

To study BCR signaling in Tec−/− B cells, we measured tyrosine phosphorylation of PLCγ2 at position Y759 (pPLCγ2), which is a major phosphorylation target of Btk and Tec (21) by flow cytometry. We found no difference in pPLCγ2 between WT and Tec−/− cells upon anti-IgM stimulation after 5 min (peak of pPLCγ2 expression, Fig. 2A) or for up to 60 min (Fig. 2B), with either a suboptimal low (4 μg/ml) or an optimal high (25 μg/ml) of anti-IgM. Accordingly, calcium influx, which occurs downstream of PLCγ2, was not increased in Tec−/− B cells compared with WT B cells. Furthermore, there was no change in Btk dependence of calcium influx, as shown by in vivo treatment with ibrutinib, a Btk inhibitor that irreversibly binds to the Btk kinase domain, thereby inhibiting its function (22) (Fig. 2C). Additionally, we found no differences in pBtk (Y223) or Btk protein levels (data not shown).

**pAkt and pS6 expression are increased in Tec−/− B cells upon BCR and CD40 stimulation**

As we did not detect differences in calcium influx, we next measured serine phosphorylation of Akt (S473), which is also activated downstream of BCR signaling, but in a pathway separate from PLCγ2, as well as phosphorylation of S6 kinase (S240/244), a downstream target of Akt.

Kinetics experiments with different stimuli showed an early peak of pAkt and pS6 expression in B cells after 1–3 h, but a higher second peak after 24 h of stimulation (Supplemental Fig. 2). pAkt expression was increased after 24 h of stimulation with both a low dose (4 μg/ml) and a high dose (25 μg/ml) of anti-IgM in Tec−/− B cells, compared with WT cells (Fig. 3A). Addition of rIL-4 to a low dose of anti-IgM did not induce differences in pAkt expression; however, both a low dose of anti-CD40 (4 μg/ml) and a high dose (20 μg/ml) in combination with rIL-4 induced robust up-regulation of pAkt expression, which was higher in Tec−/− B cells than in WT B cells (Fig. 3A). Correspondingly, phosphorylation of S6 was also increased in Tec−/− B cells compared with WT B cells following 24 h of BCR or anti-CD40 with rIL-4 stimulation (Fig. 3B). These data show that, unlike the PLCγ2/calcium flux pathway, loss of Tec expression leads to enhanced activation of the Akt/S6 pathway.

**Increased Akt and ribosomal S6 phosphorylation in Tec−/− B cells is dependent on Btk kinase activity**

As we found increased activation of the Akt/S6 pathway, but not of the PLCγ2/Ca2+ pathway, in Tec−/− B cells, we hypothesized that Tec competes with Btk in the Akt pathway. In this model, Tec kinase would be less effective in activating Akt and S6, but not involved in the PLCγ2 pathway when Btk is present.

To test this hypothesis, we performed phosphoflow analysis on B cells stimulated with anti-IgM (4 μg/ml) and rIL-4, in the absence or presence of ibrutinib in vitro (Fig. 3C), or on B cells from mice that were treated in vivo for 3 h prior to the experiment and stimulated with anti-IgM (25 μg/ml) in vitro (Fig. 3D). This allowed us to study the effect of Btk kinase activity on the Akt pathway in a setting where mature B cells are present, as opposed to Tec/Btk−/− mice in which mainly IgMhiIgDlo immature/transitional B cells are present. After 24 h in culture, pAkt and pS6 expressions were higher in Tec−/− B cells compared with WT B cells (Fig. 3C, 3D). After ibrutinib treatment in vitro, pAkt and pS6 expression decreased to similar levels in Tec−/− and WT B cells (Fig. 3C). Furthermore, after in vivo treatment, pAkt decreased in Tec−/− B cells and pS6 expression dropped to placebo-treated WT levels (p = 0.69; Fig. 3D).

In summary, these data show that in the absence of Tec, phosphorylation of Akt and ribosomal S6 is increased, whereby this increase is dependent on Btk kinase activity. We conclude that under WT conditions, Tec and Btk may compete, possibly for binding with Akt, but that Tec is less capable of efficiently activating the Akt pathway.

**Increased activation of Tec−/− B cells in vivo after immunization with model Ags**

To study the effects of increased BCR-mediated activation of Tec−/− B cells in vivo, we immunized WT and Tec−/− mice with model Ags. First, we performed a T cell–independent DNP-Ficoll immunization. In Tec−/− mice, DNP-specific IgM and IgG3 Abs in serum were both significantly increased 7 d after immunization, compared with WT mice (Fig. 4A). Next, we immunized mice with the T cell–dependent TNP-KLH Ag in alum. Seven days after the boost (day 42 after primary immunization), we found that both total and high-affinity anti-TNP Abs were significantly increased in Tec−/− mice (Fig. 4B). Ratios between high-affinity and total anti-TNP Abs were comparable between Tec−/− and WT mice (data not shown), indicating that affinity maturation was not affected in Tec−/−...
mice. Absolute numbers of germinal center (GC) B cells and follicular Th (Tfh) cells (CD3⁺CD4⁺CXCR5⁺PD1hi) in the spleen on day 42 were not different between Tec⁻/⁻ and WT mice (Fig. 4C and data not shown). However, absolute numbers of total IgM and IgG plasma cells (PC) were significantly increased in the spleens, but not BM, of Tec⁻/⁻ mice, suggesting...
that only short-lived but not long-lived PC were affected in Tec^−/−

mice (Fig. 4C).

Because Tec is also expressed in dendritic cells and T cells, we
desired to determine whether B cell–intrinsic effects of Tec defi-
cency contributed to the increase in TNP-specific Ab formation
and the increase in splenic PC upon in vivo immunization. To this
end, we cultured MACS-purified B cells for 4 d with decreasing
doses of LPS (mimicking a T cell–independent stimulus) or LPS
and rIL-4 (mimicking T cell costimulation) to induce IgM-, IgG3-,
and IgA-expressing plasmablasts, respectively. Proportions of
IgM+ plasmablasts were comparable upon LPS stimulation, but
proportions of IgG3+ plasmablasts were significantly increased in
Tec^−/− cultures with a suboptimal LPS (25 ng/ml) concentration,
and IgG1+ plasmablasts were significantly increased in Tec^−/−
cultures even at high LPS concentrations (Fig. 4D).

Taken together, these data show that B cell activation in vivo is
enhanced in Tec^−/− mice, both during a T cell–independent and a
T cell–dependent B cell response, whereby B cell–intrinsic effects
of loss of Tec (at least partially) contribute to the enhanced B cell
responses.

Higher antivirus-specific Ab levels in serum upon influenza
infection in Tec^−/− mice

To study B cell responses in Tec^−/− mice in a more physiological
setting, we performed an infection with H3N2 influenza virus
(X31 strain) (17) in Tec^−/− and WT mice. Weight loss after
infection was moderately decreased in Tec^−/− mice (Supplemental
Fig. 3A). At day 25 postinfection, PC and GC B cells, marking the
formation of inducible BALT, were found in the lungs of WT mice
(Fig. 5A). Although some Tec^−/− mice showed a slight increase in
GC B cells, there was no difference in PC numbers in the lungs
(Fig. 5A). PC and GC B cell numbers in the draining lymph nodes
were also comparable between WT and Tec^−/− mice at 7 or 15 d
postinfection (Fig. 5A). Accordingly, histological analysis of in-
ducible BALT formation in the lungs 25 d postinfection showed
no significant differences in number or size of GL7+ B cell
clusters (Fig. 5B).

Next, we measured serum Ab titers for X-31 and heterotypical Abs
directed against related H3N2 influenza viruses (England/72, Port
Chalmers/73, and Victoria/75). Both 15 and 25 d postinfection
we found higher titers of X-31–specific Abs and heterotypical Abs in
serum from Tec^−/− compared with WT mice (Fig. 5C), but not in
total serum Abs (Supplemental Fig. 3B). Because we previously
found that increased Btk activity in B cells is associated with local
autoantibody production during influenza infection (10), we per-
formed HEp2 reactivity analyses. We found no IgM autoantibodies
in the bronchoalveolar lavage fluid and detected low levels of IgG
autoantibodies in both WT and Tec^−/− mice (Supplemental Fig. 3C).

These findings indicate that both the X-31 influenza virus–specific
humoral response and the non-X31–specific antiviral response is en-
hanced in Tec^−/− mice, suggesting that B cells are more activated in
Tec^−/− mice upon influenza virus infection.

Aging Tec^−/− mice develop a spontaneous mild autoimmune
phenotype

To investigate whether the enhanced BCR signaling in the absence
of Tec is associated with autoimmunity, we aged WT and Tec^−/−

mice up to ∼34 wk and analyzed the mice for signs of B cell
activation and autoantibody formation.

Although we did not find differences in the numbers of GC
B cells in the spleens of Tec^−/− mice (Fig. 6A), the costimulation
markers CD80 and PDL1 (but not CD86) were upregulated on
these cells compared with WT controls, suggesting that they were
more activated. Accordingly, we found a significant increase in
IgM+, IgG+, and IgA+ PC in the spleens from Tec^−/− mice
(Fig. 6B). Immunohistological analysis confirmed the increase in
PC, which were present in the white pulp in the spleens of Tec^−/−

mice, but no histological abnormalities were observed.
Already at 10 wk of age, IgG1 and IgG2c Ab levels in serum of Tec\(^{-/-}\) mice were slightly increased compared with WT mice (Supplemental Fig. 4). In aged mice, we found higher serum levels of DNA-specific IgM Abs in Tec\(^{-/-}\) than in WT mice, and more Tec\(^{-/-}\) mice showed DNA-specific IgG Abs (5 of 17 Tec\(^{-/-}\) versus 1 of 22 WT) (Fig. 6C). In HEp2 reactivity assays, 13 of 21

**FIGURE 3.** Increased activation of the Akt/S6 pathway in Tec\(^{-/-}\) mice. (A) Histogram overlays (top) and quantification (bottom) of phospho-Akt expression in unstimulated B cells or following 24 h of stimulation with the indicated stimuli. (B) Histogram overlays (top) and quantification (bottom) of phospho-S6 expression in unstimulated B cells or following 24 h of stimulation with the indicated stimuli. (C) Phospho-Akt and phospho-S6 expression in B cells upon 24 h in vitro stimulation with 25 μg/ml F(ab\(^9\))\(_2\) anti-IgM, with or without ibrutinib, shown as histogram overlays (left) and quantification (right). (D) Phospho-Akt and phospho-S6 expression in B cells (from mice 3 h after in vivo treatment with ibrutinib) upon in vitro stimulation with 25 μg/ml F(ab\(^9\))\(_2\) anti-IgM, shown as histogram overlays (left) and quantification (right). Graphs represent one to four individual experiments, and n = 4–12 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 by Mann–Whitney U test.
sera from Tec−/− mice showed IgM staining, whereas IgM autoantibodies were not detectable in WT mice (Fig. 6D). For IgG autoantibodies, we found staining in 2 of 16 WT mice but in 15 of 21 Tec−/− mice (Fig. 6D).

The presence of GC and IgG autoantibodies suggested involvement of T cells in the autoimmune phenotype in Tec−/− mice. However, we found no significant differences in memory phenotype of CD4+ T cells (CD44hiCD62Llo), proinflammatory cytokine production by CD4+ T cells (IFN-γ, IL-4, IL-6), Tfh cell numbers (CXCR5hiPD1hi), or ICOS expression by Tfh cells (data not shown). Although kidneys from Tec−/− mice showed slightly more IgM and IgG immune deposits in the glomeruli, we did not find lymphocyte infiltrates in kidneys, lungs, or salivary glands in these mice (data not shown).

Taken together, these data show that aging Tec−/− mice develop a spontaneous mild autoimmune phenotype.

**TEC expression levels correlate with BTK expression levels in chronic lymphatic leukemia B cells**

To study the relationship between TEC and BTK in human B cells, we sorted three peripheral B cell subsets from healthy donors, that is, naive mature B cells (CD19+CD27−IgD+), natural effector B cells (CD19+CD27−IgM+), and IgG memory B cells (CD19+CD27+IgG+), and measured TEC and BTK RNA expression by quantitative PCR. We found expression of TEC and BTK in all three B cell subsets, whereby the lowest TEC expression was seen in natural effector B cells (Fig. 7A).

Next, we analyzed B cells from chronic lymphatic leukemia (CLL) patients, as BTK plays a crucial role in survival of these cells (7). In CLL cells, which most closely resemble natural effector B cells, expression of BTK and TEC was correlated (Fig. 7B). In most patients, the expression of BTK and TEC was higher than in natural effector B cells from healthy controls, particularly in unmutated CLL patients. Finally, we measured BTK and TEC RNA expression in several human tumor B cell lines and found that in part of the cell lines BTK and TEC expression correlated (high in RAJI and RS4;11 and low in LP1 and JVM-2; Fig. 7C). However, in the BTKlo plasma cell leukemia U226 (23) substantial TEC expression was found, and the BTKhi precursor B cell acute lymphoblastic leukemia BV173 TEC expression was very low (Fig. 7C).

These data indicate that BTK and TEC expression correlated in human CLL, but that in leukemia cell lines the two genes may be differentially expressed.

**Discussion**

Previous studies have shown that Btk and Tec may have redundant roles in B cell activation, and that Tec can partially compensate for loss of Btk (5). In this study, we show that in pre-BCR signaling,
but not in BCR signaling, lack of Tec could be completely compensated by Btk. We provide evidence that loss of Tec leads to increased B cell activation through enhanced activation of the Akt pathway, whereby increased phosphorylation is dependent on Btk kinase activity. Tec-deficient mature B cells show increased activation, proliferation, and survival upon anti-IgM and anti-CD40 with rIL-4 stimulation in vitro. Moreover, Tec-deficient mice have enhanced humoral immunity upon immunization or viral infection and develop a mild autoimmune phenotype upon aging. Importantly, these data provide evidence supporting a role for Tec in limiting B cell activation through competition with Btk.

**FIGURE 5.** Enhanced humoral immunity in Tec−/− mice after influenza infection. (A) IgM and IgG PC and GC B cell numbers in lungs and mediastinal lymph nodes (MLN) on days 7, 15, and 25 after influenza infection in the indicated mouse groups. GC B cells were characterized as CD19+IgD−CD95+, IgM− PC as CD11b+CD138−IgM+, and IgG− PC as CD11b−CD138−IgM−IgG+. (B) Representative immunohistochemical analysis of CD138+ plasma cells (blue) and GL7+ GC B cells (brown), counterstained with hematoxylin in lungs from WT and Tec−/− mice. Scale bars, 200 μm. (C) Hemagglutinin inhibition assay titers for X-31, Eng/72, P Ch/73, and Vict/75 virus-specific Abs in serum of mice 15 or 25 d postinfection. n = 8–10 per group. *p < 0.05, **p < 0.01 by Mann–Whitney U test.
PLCγ2 phosphorylation or Ca2+ influx, but it increased BCR signaling through enhanced activation of the Akt pathway. This activation was dependent on Btk kinase activity, as ibrutinib strongly reduced phosphorylation of Akt and ribosomal S6. We found no increase in Btk protein levels or in phosphorylation of Btk at the Y223 autophosphorylation site. Therefore, we propose a model in which competition for binding of downstream signaling molecules between Btk and Tec limits B cell activation in WT B cells. In line with this, we found that transgenic mice with B cell–specific overexpression of Btk protein (10) show increased phosphorylation of Btk at Y551 in the kinase domain and increased phosphorylation of S6 kinase (O.B. Corneth and R.W. Hendriks, unpublished observations). The finding that the effects of loss of Tec were limited to the Akt/S6 pathway, and not observed in the PLCγ2/Ca2+...
pathway, clearly indicates a differential role for Tec in these two pathways in which Btk acts as a key player.

It has been demonstrated that Btk can directly interact with and phosphorylate Akt in DT40 chicken B cells (8). However, phosphorylation of Akt has also been shown to depend on PI3K and Syk, but it does not require Btk (24, 25). Additionally, it was reported that in mature B cells Btk can enhance PI3K activity by bringing phosphatidylinositol-4-phosphate 5-kinase to the plasma membrane and stimulating the production of the PI3K substrate phosphatidylinositol 4,5-bisphosphate by phosphatidylinositol-4,5-bisphosphate 5-kinase (26). Through this indirect mechanism, Btk would have the capacity to enhance the activity of PI3K and thus phosphorylation of the downstream target Akt. However, this mechanism is not very likely, because the stimulation of phosphatidylinositol 4,5-bisphosphate production by Btk was shown to be independent of its kinase activity (26), and we show that increased phosphorylation of Akt and S6 in Tec−/− cells is dependent on Btk kinase activity. This is in line with published data showing downregulation of Akt phosphorylation upon ibrutinib treatment in vitro in several lymphoma and in vivo in CLL patients (27–31). These data support the model that Tec and Btk compete for binding of Akt, but that activation of Akt is more efficient by Btk.

Model Ag immunizations showed that humoral immunity was enhanced in Tec−/− mice, but affinity maturation in the GC was not affected. Additionally, we found no increase in long-lived PC in the BM, suggesting that selection into the memory compartment is normal. Increased activation of Akt promotes cell proliferation and survival (32). Indeed, we found that proliferation upon BCR stimulation was increased in Tec−/− B cells. It is likely that more B cells, including autoreactive B cells, survive in Tec-deficient mice, leading to enhanced humoral immunity and development of a mild autoimmune phenotype. As our mice are fully Tec deficient, we cannot exclude that the lack of Tec in other cells, such as T cells, contributes to the development of autoimmunity. However, we did not find any apparent differences in CD4+ T cell cytokine production or Tfh differentiation. Additionally, we found enhanced plasmablast formation in a pure B cell culture upon LPS and rIL-4 stimulation, but not with LPS alone. Moreover, we noticed that the absence of Tec made B cells quite sensitive to IL-4, as IL-4 had marked effects on activation marker expression and cell size increase of anti-igM-stimulated Tec−/− B cells (Fig. 1). We therefore conclude that the phenotype observed in Tec−/− mice is likely driven in part by a B cell–intrinsic increase in activation. The lack of a clear B cell phenotype in Tec-deficient mice on a C57BL/6 × 129 background in previous studies (5) may be attributed to the mixed background of those mice. We have previously shown that transgenic mice overexpressing human BTK only displayed an autoimmune phenotype when backcrossed onto the C57BL/6 background (10).

These data provide new insight in the role for Tec in B cell activation. We show that Tec is involved in the Akt pathway, where it may compete with Btk, but is not involved in activation of the PLCγ2 pathway. This is of particular interest in light of the current efforts to develop blocking agents that affect B cell activation in B cell malignancies. Loss or downregulation of Tec may contribute to the development of tumors through enhanced activation of Akt. Ibrutinib has shown excellent therapeutic efficacy in CLL and other B cell malignancies (33, 34). However, acquired resistance to ibrutinib, involving mutations of the ibrutinib binding site or in the Plcg2 gene, has been reported (35, 36). In the context of possible resistance to ibrutinib in B cell malignancies including CLL and potential detrimental effects of Tec cross-inhibition, it was important to also explore Tec expression in human B cells. However, we found no evidence for downregulation or dramatic increases in Tec expression in CLL B cells. Rather, BTK and TEC mRNA expression levels were somewhat increased, compared with naive B cells, and were correlated. Although second-generation Btk inhibitors with promising efficacy profiles have been developed (37, 38), it is expected that combination regimens will result in long-lasting responses, as they may overcome resistance. Indeed, combining ibrutinib with a PI3K or Akt pathway inhibitor was shown to have synergistic effects (36, 39–42).

In summary, our data showing that in primary mouse B cells Tec kinase acts to limit the activating capacity of Btk contributes to a better understanding of the differential roles of Btk and Tec. This is important for the development and improved application of BCR signal-blocking agents in the treatment of B cell malignancies as well as autoimmune disorders.

Acknowledgments
We thank Alice Muggen and Ruud Meijers for excellent technical assistance and Prof. Hermann Eibel (University Medical Center Freiburg) for help with optimizing phosphoflow.

Disclosures
The authors have no financial conflicts of interest.
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