Premature Terminal Differentiation Protects from Deregulated Lymphocyte Activation by ITK-Syk

Martina P. Bach, Eva Hug, Markus Werner, Julian Holch, Clara Sprissler, Konstanze Pechloff, Katja Zirlik, Robert Zeiser, Christine Dierks, Jürgen Ruland and Hassan Jumaa

*J Immunol* 2014; 192:1024-1033; Prepublished online 27 December 2013;
doi: 10.4049/jimmunol.1300420
http://www.jimmunol.org/content/192/3/1024

Supplementary Material  http://www.jimmunol.org/content/suppl/2013/12/26/jimmunol.1300420.DCSupplemental

References  This article cites 55 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/192/3/1024.full#ref-list-1

Subscription  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Premature Terminal Differentiation Protects from Deregulated Lymphocyte Activation by ITK-Syk

Martina P. Bach,*† Eva Hug,† Markus Werner,*† Julian Holch,‡ Clara Sprissler,§ Konstanze Pechloff,§† Katja Zirlik,§ Robert Zeiser,§ Christine Dierks,§ Jürgen Ruland,§† and Hassan Jumaa*†

The development of hematopoietic neoplasms is often associated with mutations, altered gene expression or chromosomal translocations. Recently, the t(5, 9)(q33;q22) translocation was found in a subset of peripheral T cell lymphomas and was shown to result in an IL-2–inducible kinase–spleen tyrosine kinase (ITK-Syk) fusion transcript. In this study, we show that T cell–specific expression of the ITK-Syk oncogene in mice leads to an early onset and aggressive polyclonal T cell lymphoproliferation with concomitant B cell expansion and systemic inflammation by 7–9 wk of age. Because this phenotype is strikingly different from previous work showing that ITK-Syk expression causes clonal T cell lymphoma by 20–27 wk of age, we investigated the underlying molecular mechanism in more detail. We show that the reason for the severe phenotype is the lack of B-lymphocyte–induced maturation protein-1 (Blimp-1) induction by low ITK-Syk expression. In contrast, high ITK-Syk oncogene expression induces terminal T cell differentiation in the thymus by activating Blimp-1, thereby leading to elimination of oncogene-expressing cells early in development. Our data suggest that terminal differentiation is an important mechanism to prevent oncogene-expressing cells from malignant transformation, as high ITK-Syk oncogene activity induces cell elimination. Accordingly, for transformation, a specific amount of oncogene is required, or alternatively, the induction of terminal differentiation is defective. The Journal of Immunology, 2014, 192: 1024–1033.

Spleen tyrosine kinase (Syk) is a nonreceptor tyrosine kinase that possesses two N-terminal Src homology 2 domains and a C-terminal kinase domain. Syk is widely expressed in all hematopoietic cells, mediates various biological functions, and plays a central role in B cell development and signal transduction downstream of the BCR (1–4). In T cells, Syk is mainly expressed at the double-negative and double-positive (DP) developmental stages, but its expression is markedly reduced in later stages of T cell development (5, 6). Deregulated Syk expression or activity has been frequently linked to distinct hematological and nonhematological malignancies and seems to play an important role in the development and maintenance of B cell lineage neoplasms. For instance, inhibition of Syk induces apoptosis of chronic lymphocytic leukemia and diffuse large B cell lymphoma cells, indicating that Syk is crucial for the survival of these cells (7, 8). Syk has also been linked to malignancies of other hematopoietic lineages such as peripheral T cell lymphomas (PTCLs), in which Syk is overexpressed in >90% of all PTCLs (9).

The gene encoding Syk has also been shown to be affected by chromosomal translocations. Chromosomal translocations are thought to play a major role in the development and pathogenesis of various malignancies. For example, the chromosomal translocation t(9, 12)(q22;p12) was identified in a patient with myelodysplastic syndrome and results in the expression of the TEL-Syk fusion protein, leading to constitutive activation of Syk and the ability to transform various cell lines and induce leukemia in mice (10–12). Recently, an additional chromosomal translocation involving the Syk gene, the t(5, 9)(q33;q22), was found in ~17% of unspecified PTCLs, which represent a heterogeneous and aggressive group of non-Hodgkin lymphomas (13). This translocation fuses Syk to the IL-2–inducible kinase (ITK) and results in an ITK-Syk fusion transcript. ITK is a member of the Tec kinase family, predominantly expressed in T cells and involved in normal T cell development and activation. Mice deficient for ITK show a partial block in T cell development and impaired signaling downstream of the TCR (14). Upon TCR activation, ITK is recruited to the membrane in proximity of the TCR, where it is phosphorylated and interacts with various substrates including linker for activation of T cells, SLP-76, Grb2, and phospholipase Cγ1 (PLCγ1) (15). ITK localization to the plasma membrane is mediated by the pleckstrin homology (PH) domain of ITK and is dependent on PI3K activity that converts phosphatidylinositol-(4, 5)-bisphosphate to phosphatidylinositol-(3,4,5)-trisphosphate, the ligand for the PH domain. Once activated, ITK phosphorylates PLCγ1, which induces calcium mobilization and the activation of downstream pathways regulating the activation and effector function of T cells (15). The ITK-Syk fusion transcript contains the PH domain, the Tec ho-
mology domain of ITK, and the kinase domain of Syk. It was demonstrated that this protein exhibits kinase activity and is able to phosphorylate various important signaling proteins such as PLCγ1, Akt, and Erk1/2 (16, 17).

To study the role of the ITK-Syk oncogene in disease development, we generated a mouse model for conditional ITK-Syk expression. We found that T cell–specific ITK-Syk expression leads to a severe disease development with an early onset and aggressive polyclonal lymphoproliferation by 7–9 wk of age, which is strikingly different from available data showing that T cell–specific ITK-Syk expression results in clonal T cell proliferation by 20–27 wk of age (17). In this study, we characterize the underlying mechanism for the early onset of disease and show that reduced oncogene expression prevents cell elimination by a cell-autonomous mechanism.

Materials and Methods

Mice

To generate the ITK-Syk mice, the human ITK-Syk cDNA (Supplemental Fig. 1A) (13) was cloned into a ROSA26 targeting vector (18), linearized, and electroporated into W4 embryonic stem cells obtained from 129S6/SvEv mice. Homologous recombination in the ROSA26 locus was confirmed by PCR screening (forward, 5′-CATCAGAGACCTGCTGCTA-3′; reverse, 5′-GGACAGGATAAGTATGCCATCA-3′) and Southern blot analysis. Southern blots from HindIII-digested embryonic stem cell genomic DNA were hybridized with a 5′-probe (19) and analyzed by phoshorimaging. Two clones were injected into blastocysts obtained from C57BL/6 mice and transferred back into foster females to obtain chimeric mice. The resulting ROSA26-ITK-Syk mice were crossed to either CD4-Cre mice (20) or Lck-Cre mice (21). Mice were checked daily and subsequently sacrificed and analyzed once the first prognostic symptoms were observed. All mice were bred at the animal facility of the Max Planck Institute of Immunobiology and Epigenetics. Animal experiments were done in compliance with the guidelines of German law.

Human subjects

All human tissue samples were collected after approval by the ethics committee of the Albert-Ludwigs University Freiburg (protocol number: 267/11) and after written informed consent. Defined as positive for ITK-Syk were those samples showing a Syk-specific protein band at the expected size.

Plasmids and retroviral transduction

Retroviral constructs were generated by cloning the human ITK-Syk cDNA together with flanking sequences leading to low and high ITK-Syk expression into a retroviral vector backbone containing an internal ribosome entry site–GFP sequence (pMIG). For retroviral transductions, viral supernatants were produced using the Phoenix retroviral producer cell line together with flanking sequences leading to low and high ITK-Syk expression. For retroviral transductions, viral supernatants were produced using the Phoenix retroviral producer cell line and GeneJuice (EMD Millipore) transfection reagent. Retroviral constructs were generated by cloning the human ITK-Syk cDNA with an entry site–GFP sequence (pMIG). For retroviral transductions, viral supernatants were produced using the Phoenix retroviral producer cell line and GeneJuice (EMD Millipore) transfection reagent. Retroviral supernatants were harvested after 48 h, subsequently mixed with Beko pre-T cells or freshly isolated wild-type (wt) thymocytes, and centrifuged at 300 × g at 37°C for 2 to 3 h.

Culture conditions

Primary cells were grown in Iscove’s medium (Biochrom) containing 10% heat-inactivated FCS (PAN-Biotech), 10 mM L-glutamine (Life Technologies), 50 mM 2-ME. Cells were incubated at 37°C for 24 h. Transduced pre-T cells were sorted with a MoFlo (DakoCytomation) cell sorter. For isolation of splenic T cells, splenocytes were stained with anti-Thy1.2-biotin (BD Biosciences) and FITC-conjugated streptavidin (BD Biosciences). FACS analysis was performed with FACS Calibur or LSR II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

Western blot analysis

Transduced pre-T cells were sorted with a MoFlo (DakoCytomation) cell sorter. For isolation of splenic T cells, splenocytes were stained with anti-Thy1.2-biotin (BD Biosciences) before incubation with streptavidin–biotin–HRP–conjugated magnetic beads (Miltenyi Biotec). Purification was performed with AutoMACS (Miltenyi Biotec). Cells were lysed in RIPA lysis buffer containing 50 mM Tris-HCl, 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.5 mM NaN3, 5 mM NaF, and protease inhibitor mixture (Sigma-Aldrich). Samples were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked in 5% milk powder in PBS with 0.1% Tween 20. Primary Abs were diluted in PBS with 0.1% Tween 20 supplemented with 2% BSA fraction V (Biomol) and 0.1% sodium azide (Sigma-Aldrich). Immunoreactive proteins were detected using a chemoluminescence detection system (ECL). Anti-hSyk (4D10; Santa Cruz Biotechnology), anti-CD3e (BD Biosciences), anti-CD19, and anti-IgM, anti-IgD, anti-CD44, anti-CD62L, anti-human Syk (hSyk; 4D10; Santa Cruz Biotechnology), anti-p-ERK1/2 (Thr202/Tyr204, Cell Signaling Technology), anti-p-p38 (Ser177, Cell Signaling Technology), anti-p-ZAP70 (Tyr207, Cell Signaling Technology), anti-p-PLCγ1 (Y469; Cell Signaling Technology), and anti-Blimp-1-CRD (23/11-N20; Santa Cruz Biotechnology). An APC labeling kit (AbD Serotec) or secondary Ab (anti-rabbit DyLight-649; AbD Serotec) was used in case of unlabeled primary Abs. If not stated otherwise, Abs were obtained from BD Biosciences and eBioscience. Intracellular staining was performed using FIX&PERM Cell Permeabilization Kit (An Der Grub Bio Research, Klumern, Austria). FACS analysis was performed with FACSCalibur or LSR II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

ELISA and cytometric bead array analysis

Serum of mice was collected using serum collection tubes (Microtainer; BD Biosciences), and Ig titers were determined by ELISA. All Abs were obtained from Southern Biotechnology Associates. Secondary Abs were AP-labeled using 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich) as detection reagent.

Cytokine levels were analyzed using a cytometric bead array (CBA) mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences). For in vitro cytokine detection, splenic T cells were isolated by negative selection with magnetic beads (Miltenyi Biotec). Cells were either left untreated or stimulated with 3 μg/ml anti-CD3e and 10 μg/ml anti-CD28 (eBioscience). Supernatants were harvested after 20 h and subjected to CBA analysis. Results were calculated by FACSCalibur or LSR II (BD Biosciences). Data were analyzed using FCAP Software (Soft Flow).

Real-time PCR analysis

Total RNA was isolated using Quick-RNA Mini Kit (Zymo Research) and cDNA was reverse transcribed with M-MLV Reverse Transcriptase (Promega) or Qiagen QuantiTect reverse transcriptase (Qiagen). Gene expression was determined by RT-PCR using Bio-Rad iCycler (Bio-Rad). Gene expression was determined byqRT-PCR using the CFX96 system (Bio-Rad). Primer sequences were obtained from Southern Biotechnology Associates. Gene expression was determined by threshold cycle method.

Histology and immunofluorescence

For immunohistochemistry, mouse tissues were fixed in formalin, and paraffin-embedded sections were stained with H&E or overnight with anti-CD3 (Abcam) primary Ab followed by a 30-min incubation with HRP-coupled secondary Abs. Immunodetection was performed using 3,3′-diaminobenzidine (EnVision+System-HRP, DakoCytomation). Photographs were taken with Axioplan 2 (Zeiss). For immunofluorescence, cells (5 × 104 cells/chamber) were seeded into eight-chamber slides and fixed with 2% paraformaldehyde for 15 min at room temperature, followed by permeabilization with 0.5% saponin and 1% BSA in PBS for 30 min. After blocking, cells were subjected to immunofluorescence staining with anti-hSyk (4D10; Santa Cruz Biotechnology) and anti-mouse Cy3 (Invitrogen). Cells were examined by fluorescence microscopy with the LSM 780 confocal microscope (Zeiss).

Genscan analysis

Genomic DNA from purified primary total lymphocytes was analyzed for clonal TCR gene rearrangements on the TCR-β locus. TCR-β gene clonality was reassayed at VD-J and incomplete VD-J rearrangements in five different PCR reactions: Vβ (1–20)–Jβ1, Vβ (1–20)–Jβ2, DJβ1–Jβ1, DJβ2–Jβ2, and DJβ1–Jβ2. The primers used for detection of gene recombination consist of a mixture of 20 family-specific upstream primers located within

The Journal of Immunology 1025

Downloaded from www.jimmunol.org by guest on May 2, 2017
Vβ gene segments, consensus primers immediately located 5’ of Dβ1/Dβ2, and 5’ FAM-labeled consensus downstream primers immediately located 3’ of Jβ1/Jβ2 as described previously (24, 25). 5’-FAM-labeled PCR products were size separated by capillary POP-7-Polymer electrophoresis and visualized via automated scanning using a 3700 Genetic Analyzer (Applied Biosystems). All reagents were obtained from Applied Biosystems. Fragment size distribution was determined by GeneMapper software (Applied Biosystems) and analyzed as previously described (26, 27).

Statistical analysis

Results were analyzed for statistical significance with GraphPad Prism 4 software (GraphPad) using Student t test or Mann–Whitney U test.

Results

Generation of mice with T cell–specific ITK-Syk oncogene expression

To study the effect of the ITK-Syk oncogene in vivo, we generated a mouse model for conditional expression of the ITK-Syk fusion protein in T cells. We introduced the cDNA for the human ITK-Syk fusion protein isolated from a PTCL patient harboring the t(5, 9) (q33;q22) translocation together with a loxP-flanked transcriptional STOP cassette into the ubiquitously expressed ROSA26 locus (Supplemental Fig. 1) (13). Cre-mediated excision of the STOP cassette allows ITK-Syk expression under the control of the ROSA26 promoter (Supplemental Fig. 1B). To express the ITK-Syk fusion protein specifically in T cells, the mice were crossed to either CD4-Cre (20) or Lck-Cre (21) mice. Because both mouse strains showed the same phenotype, we summarized both as ITK-Syk mice. T cell–specific ITK-Syk expression was confirmed by immunoblot and FACS analysis of splenic T cells (Fig. 1A, 1B).

We first analyzed the T cell compartment in 4- to 5-wk-old ITK-Syk mice. No differences in percentages or total cell numbers were detected for DP or single-positive (SP) thymocytes as well as CD4+ or CD8+ peripheral T cells in the spleen (Fig. 1C–F), suggesting that expression of the ITK-Syk oncogene does not interfere with T cell development in the thymus.

ITK-Syk mice develop early lymphoproliferation and systemic inflammation

By 7–9 wk of age, ITK-Syk mice showed first signs of malaise. Diseased ITK-Syk mice have enlarged spleens comprising an expanded T cell pool with mainly CD4+ T cell numbers being increased (Fig. 2A, 2B). Histological analysis of spleen and liver demonstrated a severe disruption of their normal architecture with altered follicular structures and widely scattered T cells in the

FIGURE 1. Normal T cell development in mice expressing the ITK-Syk oncogene in T cells. (A) Immunoblot analysis of T cell–specific ITK-Syk expression. Splenocytes were isolated from an ITK-Syk mouse and a control littermate, sorted with magnetic beads for Thy1.2+ and Thy1.2- cells, and subjected to immunoblot analysis. Data are representative of two independent experiments. (B) Specific ITK-Syk expression in CD4+ and CD8+ T cells. FACS analysis of splenocytes (gated on living cells) from an ITK-Syk mouse (red line) and a control littermate (gray filled) stained for CD4, CD8, CD19, and intracellular hSyk. Data are representative of two independent experiments. FACS analysis of thymocytes (C) and splenic T cells (D) from 4- to 5-wk-old control and ITK-Syk mice (gated on living cells) stained for the expression of CD4 and CD8. Numbers in quadrants indicate percentage of individual thymocytes subsets. Data are representative of more than three independent experiments. (E) Cell numbers from total, DP, and SP thymocytes of 4- to 5-wk-old control and ITK-Syk mice (n = 7/group) are shown. Statistical significance was analyzed using Student t test (p = 0.19 [total]; p = 0.23 [DP]; p = 0.39 [SP]). (F) Total splenic T cell numbers (Thy1.2+) from 4- to 5-wk-old control and ITK-Syk mice (n = 7/group) are indicated. Statistical significance was analyzed using Student t test (p = 0.82).
spleen (Fig. 2C, Supplemental Fig. 2A). To assess whether the expanded T cells arose from one or several T cell clones, we characterized T cell clonality by performing a PCR-based and computer-assisted fragment length analysis (Genscan) for TCR-β locus gene rearrangement (26, 27). In a clonal disease, only few rearrangements representing one or more cell clones would be predominant over a polyclonal background, whereas in polyclonal samples, many different amplification products will be detected. Diseased ITK-Syk mice showed a polyclonal TCR-β repertoire comparable to healthy control littermates (Fig. 2D). To determine whether the polyclonal expanded T cells are activated, we performed FACS analysis of splenic T cells from control and ITK-Syk mice. T cell activation is associated with an increased expression of the membrane Ag CD44 and a decreased expression of the cell adhesion molecule CD62L (28). Indeed, both CD4+ and CD8+ T cells in the spleen of ITK-Syk mice upregulated CD44 and downregulated the expression of CD62L (Fig. 2E).

Because activated T cells are an important source of cytokines during immune responses (29), we determined cytokine levels in the serum of diseased mice. We detected elevated levels of inflammatory cytokines such as IFN-γ, TNF, and IL-6 and slightly increased levels of IL-2, IL-4, and IL-10 in the serum (Fig. 3A), suggesting that ITK-Syk–mice developed systemic inflammation. To examine whether the ITK-Syk–expressing T cells secrete these inflammatory cytokines, we isolated splenic T cells from ITK-Syk and control mice and measured cytokine levels in the supernatants of in vitro cultured T cells. As in vivo, we detected significantly elevated amounts of inflammatory cytokines in the supernatants of T cells from ITK-Syk mice such as IFN-γ, TNF, IL-6, IL-2, and IL-10, whereas splenic T cells from control mice barely secreted cytokines (Fig. 3B). Upon in vitro stimulation, ITK-Syk–expressing T cells were able to secrete higher amounts of these cytokines, similar to control T cells or even significantly higher in case of IL-4 and IL-10 (Fig. 3B). This suggests that the activated ITK-Syk–expressing T cells are not anergic and are still able to react to extrinsic stimuli comparable to or even more sensitive than wt T cells. Together, these data show that peripheral ITK-Syk–expressing T cells are activated and secrete both Th1 and Th2 inflammatory cytokines that might lead to severe systemic inflammation in these mice.

**Elevated B cell numbers and Ig titers in ITK-Syk mice**

T cell–derived cytokines play a crucial role in promoting B cell activation and Ig class switch (30). Hence, the secretion of various cytokines by ITK-Syk–expressing T cells raised the question whether B cells are also affected in these mice. Interestingly, ITK-Syk mice showed increased numbers of mature B cells in the spleen possessing similar expression levels of CD19, IgM, and IgD BCRs compared with splenic B cells from control littermates (Fig. 4A, 4B). Further, we could detect an increased amount of plasma cells in the spleen of ITK-Syk mice (Fig. 4C). Thus, we determined Ig titers in the serum of diseased ITK-Syk mice and could detect elevated levels of IgM, IgG, and IgA Abs (Fig. 4D). These data suggest that the increased secretion of various cytokines by ITK-Syk–expressing T cells affects B cell numbers and Ig titers observed in ITK-Syk mice.

**Role of ITK-Syk protein expression levels**

Recently, we reported that mice with T cell–specific ITK-Syk expression show a severe reduction of total thymocytes and peripheral T cells by 4 to 5 wk, but later developed a lymphoproliferative disease by 20–27 wk with clonal T cells infiltrating all organs (17). Although the targeted locus, the targeting strategy for conditional ITK-Syk expression, and the induction of ITK-Syk...
expression by CD4-Cre transgenic mice were the same in both studies, the phenotypes are strikingly different. The expected ITK-Syk protein sequence was identical, but the sequence preceding the translational initiation site of the ITK-Syk CDNA was different in the two models. This sequence is important for the initiation of translation and thus protein production (31). Therefore, we compared the ITK-Syk expression levels in thymocytes from both ITK-Syk mouse models and found striking differences in ITK-Syk protein levels, whereas ITK-Syk RNA levels were similar (Fig. 5A, Supplemental Fig. 2B).

To investigate whether also in human PTCLs ITK-Syk protein expression levels vary, we examined the presence of ITK-Syk in 22 PTCL samples, out of which we identified 2 as positive for ITK-Syk. Interestingly, the ITK-Syk protein levels differed in these two PTCL samples, out of which we identified 2 as positive for ITK-Syk expression. These findings indicate that ITK-Syk expression levels vary, and that the presence of ITK-Syk is not limited to mouse models.

Different cellular activation by low and high ITK-Syk expression

To explain the finding that low and high ITK-Syk oncogene expression in T cells lead to different disease development in mice, we investigated the molecular mechanisms of how low and high ITK-Syk expression influence thymocyte development. We generated retroviral expression constructs for ITK-Syk carrying different flanking sequences resulting in low and high ITK-Syk protein levels when expressed in different cell lines (ITK-Syk<sub>low</sub> [IS(lo)] and ITK-Syk<sub>high</sub> [IS(hi)]), similar to the levels found in the corresponding mice (Fig. 5B, 5C). We retrovirally transduced wt thymocytes with the expression constructs for IS(hi), IS(lo), or an empty control vector and detected an upregulation of CD69 and the phosphorylation of important TCR signaling molecules such as ZAP70, PLCγ1, Akt, and Erk1/2, when ITK-Syk was highly expressed (Fig. 5D, 5E). We also investigated the subcellular localization of IS(lo) and IS(hi) and found that the majority of the ITK-Syk protein was localized at the plasma membrane in both cases (Supplemental Fig. 3). However, we also found that the increased expression of IS(hi) resulted in higher ITK-Syk protein amounts at the plasma membrane, and it is not clear whether and how this interferes with the function of other signaling molecules.

High ITK-Syk expression induces premature T cell differentiation in the thymus

Developing T cells that receive strong signals via their TCR are eliminated from the repertoire by negative selection (32). Because high ITK-Syk expression in wt thymocytes led to their activation, we investigated whether genes associated with negative selection are upregulated in thymocytes from young (4 to 5 wk of age) mice with high ITK-Syk oncogene levels [IS(hi) mice]. Compared with thymocytes from control and low ITK-Syk–expressing mice [IS(lo) mice], thymocytes from IS(hi) mice showed only a slight, but not significant, increase of BIM and NUR77 transcripts (Supplemental Fig. 4A), genes known to be key mediators of thymocyte negative selection (33, 34). This suggests that high ITK-Syk expression may trigger other signaling cascades than those activated during negative selection. Activated, Ag-experienced T cell subsets, which have undergone effector differentiation, show elevated expression of Blimp-1, a protein important for terminal differentiation of both effector T and B cells and tumor suppression in B lymphomas (35–38). Thus, we tested whether high ITK-Syk...
expression induced the expression of Blimp-1. Indeed, we found that wt thymocytes transduced with the construct for IS(hi), but not IS(lo), showed a clear induction of Blimp-1 expression (Fig. 6A). Interestingly, this could be prevented by treating IS(hi)-expressing thymocytes with the Syk inhibitor R406 (Supplemental Fig. 4B).

FIGURE 4. Diseased ITK-Syk mice have elevated B cell numbers and Ig titers. (A) Total splenic B cell (B220⁺) numbers from control and ITK-Syk mice (n = 12/group) are shown. (B) FACS analysis of splenocytes (gated on living cells) from ITK-Syk and control mice stained for CD19 versus CD3ε (left panel). CD19⁺ B cells from ITK-Syk mice show expression of IgM and IgD similar to B cells from control mice (right panel). Data are representative of more than three independent experiments. (C) ITK-Syk mice have an increased amount of plasma cells as shown by FACS analysis of splenocytes, gated on living cells, from ITK-Syk and control mice stained for B220 and CD138. Data are representative of three independent experiments. (D) ELISA analysis of serum IgM, IgG, and IgA Ig titers of control and diseased ITK-Syk mice (n = 5/group). (A and D) Statistical significance was analyzed using Student t test or Mann–Whitney U test. **p < 0.01, ***p < 0.001.

FIGURE 5. Role of different ITK-Syk protein expression levels. (A) ITK-Syk protein levels were detected by immunoblot analysis in total lysates from thymocytes isolated from a control and an ITK-Syk mouse as well as from an ITK-Syk mouse from our previous work (17). ITK-Syk expression was detected using an anti-hSyk Ab. BAP31 served as loading control. Data are representative of three independent experiments. (B) Schematic illustration of the human ITK-Syk cDNA together with the sequence context preceding the translational initiation site accounting for low [IS(lo)] and high [IS(hi)] ITK-Syk expression. (C) Beko pre-T cells were retrovirally transduced with an empty vector control (EV) and the IS(lo) and IS(hi) constructs shown in (B), sorted, and subjected for immunoblot analysis. ITK-Syk expression was detected using an anti-hSyk Ab. BAP31 served as loading control. Data are representative of three independent experiments. (D and E) High, but not low, ITK-Syk expression immediately activates wt thymocytes. FACS analysis of wt thymocytes retrovirally transduced with an empty vector control (EV; gray filled), the IS(lo) construct (red line), and the IS(hi) construct (blue line) and stained for the expression of CD69 (D) and p-ZAP70, p-PLCγ1, and p-Akt, as well as p-Erk1/2 (E). Data are representative of two independent experiments.
Based on the finding that high, but not low, ITK-Syk expression led to a strong activation of thymocytes and the induction of Blimp-1 in vitro, we tested whether also in mice, high ITK-Syk expression directly activates the thymocytes, which might induce premature T cell differentiation in the thymus. Thus, we analyzed thymocytes from young IS(hi) mice for expression of Blimp-1, and consistent with our previous data, we found elevated levels of Blimp-1 in thymocytes from IS(hi) mice compared with control mice on both the protein and RNA level (Fig. 6B, 6C). This was accompanied by thymic atrophy and a severe reduction of DP and SP thymocyte numbers as well as peripheral T cell numbers in IS(hi) mice by 4 to 5 wk (Fig. 6D, Supplemental Fig. 4C, 4D) (17). In contrast, Blimp-1 expression was not elevated in thymocytes from IS(lo) mice compared with the control, and also total thymocyte numbers were similar between control and IS(lo) mice (Fig. 6B–D).

High ITK-Syk expression activates Blimp-1 via STAT3

We next aimed to identify the signaling pathways linking ITK-Syk to the expression of Blimp-1. It is shown that STAT3 induces the expression of Blimp-1 and that Syk phosphorylates and activates STAT3 (39, 40). Therefore, we analyzed the phosphorylation status of STAT3 upon expression of IS(hi) and IS(lo) constructs in wt thymocytes and detected elevated STAT3 phosphorylation only upon high ITK-Syk expression (Fig. 7A). This suggests that high ITK-Syk expression activates STAT3, which then induces expression of Blimp-1 in thymocytes. Interestingly, Blimp-1 gene expression is further augmented by cooperation of STAT3 with IFN regulatory factor 4 (IRF-4) (41). We analyzed IRF-4 expression and found that only in thymocytes from IS(hi) mice, IRF-4 transcript levels were significantly elevated (Fig. 7B), suggesting that STAT3 and IRF-4 cooperatively induce the expression of Blimp-1. In addition, expression of the Blimp-1 antagonist B cell lymphoma-6 (Bcl-6) was selectively decreased only in thymocytes from IS(hi) mice (Fig. 7B) (42). Together, this indicates that high ITK-Syk expression leads to the induction of Blimp-1 expression via STAT3 and IRF-4, which is augmented by downregulation of the Blimp-1 antagonist Bcl-6.

These data suggested that high ITK-Syk expression in thymocytes induces Blimp-1–mediated premature terminal differentiation. Thus, Blimp-1 downregulation might be required to escape this premature terminal differentiation in the thymus. We tested this hypothesis in isolated splenic T cells from control and diseased (29-wk-old) IS(hi) mice that have developed clonal T cell lymphomas (Fig. 7C) (17). No Blimp-1 protein expression was detected in the tumors from diseased IS(hi) mice (Fig. 7D). Hence, a possible explanation for the delayed disease development in IS (hi) mice as compared with IS(lo) mice might be that the majority of T cells are deleted in the thymus by activation of Blimp-1–mediated premature terminal differentiation and that only those cells that failed to induce Blimp-1 develop tumors.

Discussion

In this study, we show that mice with T cell–specific ITK-Syk oncogene expression developed an aggressive polyclonal T cell lymphoproliferation with concomitant B cell expansion and systemic inflammation within 7–9 wk after birth. The phenotype is strikingly different from our previous model for T cell–specific ITK-Syk expression showing a profound loss of thymocytes and the development of clonal T cell lymphomas by 20–27 wk of age (17). Comparison of both mouse models revealed that the different phenotypes are caused by different expression levels of ITK-Syk.

Usually, developing T cells become positively or negatively selected depending on the strength and duration of their TCR-derived signals (43). Strong TCR-derived signals are linked to the elimination of developing T cells from the repertoire by negative selection (32). Interestingly, young IS(hi) mice showed a severe reduction of total DP and SP thymocytes, whereas the T cell development in the thymus of mice with low ITK-Syk expression was not affected. These data suggested that high ITK-Syk expression might mimic strong TCR signaling and lead to negative selection, whereas the signals induced by low ITK-Syk expression might be...
Interestingly, the expression of this so-called Blimp-1, which has a reduced function on multiple target genes (46–48), expression or additionally express a second isoform of Blimp-1, induced Blimp-1–mediated premature terminal differentiation.

ITK-Syk–expressing thymocytes have to bypass the oncogene-signaling after high ITK-Syk expression, the premature death of thymocytes is triggered by other signaling cascades than that activated during thymocyte negative selection.

Interestingly, thymocytes from IS(hi) mice showed elevated expression of Blimp-1, which is usually highly expressed in effector and memory T cell subsets and is crucial for controlling T cell homeostasis by promoting activation-induced cell death (36, 44, 45). Blimp-1–deficient effector T cells are resistant to activation-induced cell death, which leads to the accumulation of effector and memory T cells in Blimp-1–deficient mice that develop autoimmune and inflammatory symptoms (45). This suggests that Blimp-1 might play a role in the selection of early T cells and that the strong signals induced by high ITK-Syk expression trigger premature thymocyte differentiation by the activation of Blimp-1. Interestingly, the tumor-forming T cells in diseased IS(hi) mice lack expression of Blimp-1, suggesting that single T cell clones in the thymus of IS(hi) mice that fail to induce Blimp-1 escape this elimination and proliferate by strong ITK-Syk-signaling. Hence, to escape cell elimination, high ITK-Syk–expressing thymocytes have to bypass the oncogene-induced Blimp-1–mediated premature terminal differentiation. In line with this, most PTCLs were reported to lack Blimp-1 expression by activating STAT3. Consistent with this, it was reported that expression of a dominant-negative form of STAT3 inhibits Blimp-1 mRNA induction (49). Besides the activation of STAT3, high ITK-Syk expression also led to an upregulation of IRF-4 expression, which, together with STAT3, controls the expression of Blimp-1 (41). Hence, strong Syk activity mediated by high ITK-Syk expression is required for STAT3–IRF-4–mediated induction of Blimp-1 gene expression, leading to premature T cell differentiation in the thymus. Although we found Blimp-1 to be induced upon high ITK-Syk expression, we cannot exclude that additional genes are differentially expressed in the two mouse strains and that this may contribute to the elimination of high oncogene-expressing cells. Additional experiments are required to characterize these genes and the involved molecular mechanisms.

Low ITK-Syk expression in thymocytes did not lead to the induction of Blimp-1 or loss of thymocytes in IS(lo) mice. However, low ITK-Syk–expressing T cells that migrate to the periphery showed signs of activation and secreted inflammatory cytokines. The threshold at which different Ags trigger T cell activation depends on various factors including number, affinity, and kinetics of the TCR–peptide–MHC interaction and can be modulated by signals from costimulatory molecules such as CD28, CTLA-4, or LFA-1 (50–54). The finding that T cells in the periphery of IS(lo) mice are moderately activated suggests that the signals induced by low ITK-Syk expression, although not sufficient to induce terminal differentiation in the thymus, might poise the T cells for stimulation, thereby reducing the requirements for activation. This could explain the polyclonal expansion and activation of low ITK-Syk–expressing T cells that secrete inflammatory cytokines, which in turn might account for the elevated B cell numbers and serum Ig titers as well as the systemic inflammation, already in very young IS(lo) mice. Interestingly, this phenotype is similar to mice with T cell–specific elevated PI3K signaling, which also leads to both T and B cell lymphoproliferation and elevated cytokine and Ig titers (55).

Together, our data suggest that the expression level of an oncogene is crucial for the development and the course of disease. Although high ITK-Syk expression in thymocytes led to premature
T cell differentiation by induction of Blimp-1, thereby preventing the polyclonal growth of T cells, low ITK-Syk oncogene expression did not induce Blimp-1-mediated elimination of oncogene-expressing cells. This results in a severe and early onset of lymphoproliferation with systemic inflammation. In a physiological situation, both genetic and epigenetic modifications influence promoter activity and may thus influence the expression level of an oncogene. However, the t(5;9)(q33;q22) translocation resulting in the ITK-Syk fusion transcript was only characterized by fluorescence in situ hybridization and RT-PCR approaches, but no data have been available about ITK-Syk protein expression levels in patients so far. By analyzing PTCL samples, we detected different expression levels of the ITK-Syk fusion protein, indicating that the ITK-Syk protein levels may vary between individuals. However, to conclusively clarify to what extent ITK-Syk expression levels differ between individual patients and whether these differences are correlated with the course of disease, more samples need to be analyzed. Nevertheless, our results indicate that the expression level of an oncogene is a crucial parameter for the initiation and progression of human malignancies, which has to be considered for better understanding and better treatment of these diseases.

Acknowledgments

We thank B. Kanzler, N. Joswig, U. Stauffer, and C. Johner for mouse work and further assistance. We also thank A. Wurth and S. Hobitz for cell sorting and P. J. Nielsen for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


