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DISSERTATION

Studies on the role of the tyrosine kinase Itk in T cell development and function

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Verfasserin / Verfasser:	Julia Raberger
Matrikel-Nummer:	9909858
Dissertationsgebiet (lt. Studienblatt):	Genetik/Mikrobiologie
Betreuerin / Betreuer:	Univ.-Prof. Dr. Wilfried Ellmeier

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SUMMARY

Vav1 and the Tec family kinase Itk act in similar T cell activation pathways. Both molecules interact with members of the Cbl family of E3 ubiquitin ligases, and signaling defects in *Vav1*$^{-/-}$ T cells are rescued upon deletion of Cbl-b. During my PhD study I investigated the relation between Itk and Cbl-b or Vav1 by generating Itk/Cbl-b and Itk/Vav1 double-deficient mice. Deletion of Cbl-b in *Itk*$^{-/-}$ CD4^{+} T cells restored proliferation and partially IL-2 production, and led also to a variable rescue of IL-4 production. Thus, Itk and Vav1 act mechanistically similar in peripheral T cells, since the defects in *Itk*$^{-/-}$ T cells, like in *Vav1*$^{-/-}$ T cells, are rescued if cells are released from the negative regulation mediated by Cbl-b. In addition, only few peripheral CD4^{+} and CD8^{+} T cells were present in *Vav1*$^{-/-}$*Itk*$^{-/-}$mice due to severely impaired thymocyte differentiation. *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocyte numbers were strongly reduced compared to wildtype, *Itk*$^{-/-}$or *Vav1*$^{-/-}$ mice, and DP thymocytes displayed increased cell death and impaired positive selection. Therefore, our data also reveal that the combined activity of Vav1 and Itk is required for proper T cell development and the generation of the peripheral T cell pool.

Itk has also been shown to be essential for the development of conventional T cells. As a consequence, *Itk–/–* mice have a relative increase in Memory phenotype (MP) T cells with innate characteristics. We could show in an Affymetrix gene chip approach that the transcription factor PLZF is up-regulated in the *Itk–/–* T cell population. Immunoblot analysis revealed that PLZF was primarily expressed in the CD4^{+}CD44^{hi} population both in wildtype and *Itk*$^{-/-}$ T cells. This observation suggested a link between PLZF expression and MP CD4^{+} T cells. Transgenic expression of PLZF during T cell development and in CD4^{+} and CD8^{+} T cells induced a T cell intrinsic program leading to an increase in peripheral CD44^{hi} MP CD4^{+} and CD8^{+} T cells and a corresponding decrease of naïve CD44^{lo} T cells. The MP CD4^{+} and CD8^{+} T cells produced IFNγ upon PMA/ionomycin stimulation, thus showing innate-like function. Changes in the naïve versus memory-like subset distribution were already evident in SP thymocytes, indicating PLZF-induced T cell developmental alterations. In addition, CD1d-restricted NKT cells in PLZF transgenic mice showed impaired development and were severely reduced in the periphery. Finally, after anti-CD3/CD28 stimulation, CD4^{+} transgenic T cells showed reduced IL-2 and IFNγ production but increased IL-4 secretion due to enhanced IL-4 production of the CD44^{hi}CD62L^{+} subset. Our data indicate that PLZF is a novel transcriptional regulator of the development of CD44^{hi} MP T cells with a characteristic partial innate-like phenotype.

ZUSAMMENFASSUNG

Aus bisher publizierten Studien war bekannt, dass der Guanosinnukleotid-Austauschfaktor Vav1 und die Tyrosin-Kinase Itk, ein Mitglied der Familie der Tec Kinasen, in ähnlichen T-Zell Aktivierungs-Signalwegen agieren. Beide Proteine interagieren mit Cbl-b, einer E3-Ubiquitin-Ligase. Während meiner Doktorarbeit untersuchte ich das genetische Zusammenspiel dieser drei Proteine mit Hilfe von Itk/Vav1 sowie Itk/Cbl-b Doppel-knockout Mäusen. Die Abwesenheit von Cbl-b in *Itk*$^{-/-}$ CD4^{+} T-Zellen führte wieder zu einer normalen T-Zell-Proliferation und IL-2 Produktion nach T-Zell-Rezeptor-Aktivierung, während die Produktion von IL-4 nur teilweise wieder erlangt werden konnte. Diese „Rettung" des Itk-Defekts in Abwesenheit von Cbl-b ist sehr ähnlich der Beobachtung, dass der Defekt von Vav1-defizienten T-Zellen ebenfalls durch die Entfernung von Cbl-b aufgehoben werden konnte. Unsere Studien zeigen daher auf genetischer Ebene, dass Itk- und Vav1-abhängige Signalwege in T-Zellen mechanistisch ähnlich agieren bzw. durch Cbl-b ähnlich reguliert werden. Die Analyse von *Vav1*$^{-/-}$*Itk*$^{-/-}$ Mäusen hat ergeben, dass die Entstehung von T-Zellen in Abwesenheit von Itk und Vav1 stark beeinträchtig war und nur sehr wenige CD4^{+} und CD8^{+} T-Zellen vorhanden waren. Wir konnten zeigen, dass diese starke Reduktion durch einen erhöhten Zelltod von Thymozyten und einer Blockade der positiven Selektion während der Thymozytenreifung hervorgerufen worden ist. Unsere Daten zeigen daher zum ersten Mal, dass die kombinierte Aktivität von Vav1 und Itk für die Entstehung von T-Zellen und die Generierung des peripheren T-Zell-Pools notwendig ist.

Studien haben gezeigt, dass Itk für die Entstehung der sogenannten „konventionellen" T-Zellen wichtig ist. In Abwesenheit dieser Tyrosin-Kinase ist deshalb eine spezielle Population von CD44^{hi} Memory-Phänotyp (MP) T-Zellen relativ erhöht. Mittels einer Affymetrix gene chip Analyse konnten wir zeigen, dass der Transkriptionsfaktor PLZF in Itk knockout T-Zellen hochreguliert ist. Die erhöhte Expression dieses Faktors wurde auf die CD44^{hi} CD4^{+} T-Zellen zurückgeführt. T-Zellspezifische transgene Überexpression von PLZF in Mäusen induzierte ein T-Zell-intrinsisches Programm, welches zur Entstehung dieser CD44^{hi} T-Zellen führte. Die entstandenen MP CD4^{+} und CD8^{+} T-Zellen produzierten IFNγ nach Stimulierung mit PMA und Ionomycin, was eine charakteristische Eigenschaft von den sogenannten „innate-like" T-Zellen ist. Die Veränderung der Verteilung von naiven versus MP T-Zellen in den PLZF transgenen Mäusen konnte schon während der T-Zell-Entwicklung beobachtet werden. Weiter zeigten die transgenen Mäuse, aufgrund eines Entwicklungsdefektes, eine stark verringerte Anzahl von CD1d-abhängigen NKT-Zellen in

Thymus und Milz. Zudem konnten wir zeigen, dass die transgenen $CD4^+$ T-Zellen nach T-Zell-Rezeptor-Stimulierung reduzierte Mengen an IL-2 und IFNγ, jedoch erhöhte Mengen von IL-4 produzierten. Die Daten des zweiten Teils meiner Doktorarbeit weisen darauf hin, dass PLZF ein wichtiger Transkriptionsfaktor für die Reifung und Funktion von $CD44^{hi}$ MP T-Zellen mit „innate-like“ Eigenschaften ist.

1

INTRODUCTION

1.1 T lymphocytes

Mature T lymphocytes (T cells) circulate through the peripheral lymphoid tissues where they are important mediators of an adaptive immune response. T cells recognize their corresponding antigens through their surface receptors (T cell receptor, TCR) presented in the context of major histocompatibility complex (MHC) molecules by an antigen presenting cell (APC). The majority of T cells express αβ-T cell receptor complexes on their surface, composed of α- and β-chains responsible for the recognition of antigen, and the CD3γ-, CD3δ- and CD3ε-chains which mediate signal transduction. In addition a second signal is provided by simultaneous binding of the MHC molecules on the APC to the coreceptors (CD4 or CD8) on the T cell, which is necessary for proper T cell activation. The Expression of the coreceptor defines two functionally distinct populations of peripheral T cells. Most $CD4^+$ T cells are MHC class II restricted and function as helper T cells, whereas $CD8^+$ T cells are mainly MHC class I restricted and acquire a cytotoxic phenotype. Upon activation, helper T cells secrete cytokines to stimulate effector cells of the immune system, while activated cytotoxic T cells destroy their target cells via secretion of cytotoxic components.

1.1.1 T cell development

T cells develop from committed lymphoid progenitors that arise in the bone marrow and migrate to the organ of T cell development – the thymus. The thymus provides a special environment which leads to proliferation of the lymphoid precursors and to the commitment into the T cell lineage.

Development of the conventional αβ-TCR lineage: The most immature thymocytes express neither CD4 nor CD8 and are therefore termed double-negative (DN) cells and can be divided into different maturation stages due to their expression of surface molecules (1). Stage 1 cells

(DN1) express CD44 an adhesion molecule and are negative for CD25 (α-chain of the IL-2 receptor). At stage 2, when the cells up-regulate CD25 (DN2 cells) they begin to rearrange the β-chain locus and progress into stage 3, where they become $CD44^{low}$ (DN3 cells). The DN3 stage cells get arrested until they have productively rearranged their β-chain locus. If the produced β-chain pairs with the pre-α-chain (pTα) it forms the pre-T cell receptor (pre-TCR). Cell surface expression of the pre-TCR together with CD3 signals the termination of β-chain gene rearrangement (termed β-selection) and leads to excessive proliferation, resulting in the loss of CD25 (DN4-stage) . Eventually when the DN4 cells cease to proliferate, they express CD4 and CD8 and are therefore termed double positive cells (DP) (2). This small DP cells then begin with the rearrangement of the α-chain locus. After a successful rearrangement, the DP cells express low levels of a αβ-TCR and the associated CD3 complex which is necessary for positive selection. Positive selection prevents the DP cells from programmed cell death (apoptosis) and leads to their maturation into $CD4^+$ single-positive (CD4 SP) or $CD8^+$ single-positive (CD8 SP) cells. Only very few of the TCRs generated will be able to recognize self-peptide/self-MHC complexes and thus are selected for survival in the thymus. These positively selected cells will show self-MHC restricted responses to foreign antigens. T cells whose TCR recognizes self-peptide/self-MHC complexes too strongly will be negatively selected and undergo apoptosis to prevent autoimmunity, since they would be potentially self-reactive (3).

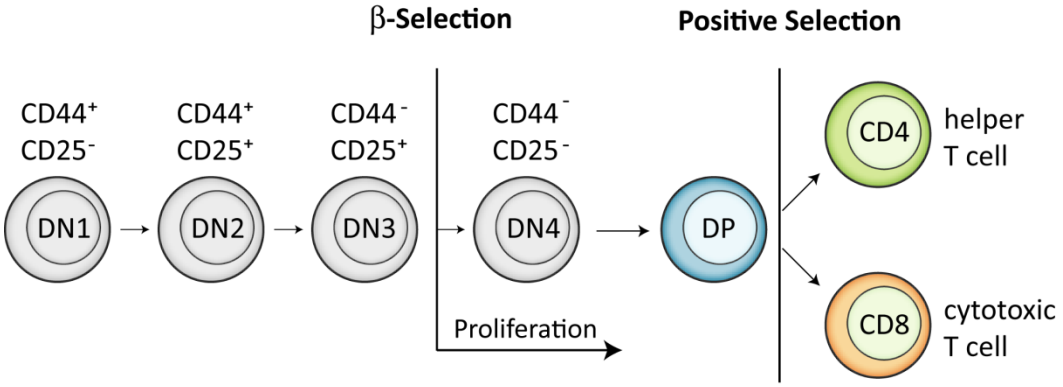

Fig.1 shows a simplified scheme of the conventional T cell development in the thymus from DN-stages on until mature $CD4^+$ or $CD8^+$ single-positive T cells.

Development of non-conventional αβ-TCR lineages: $CD4^+$ $CD8^+$ thymocytes give rise to not only the conventional T cell lineage, but also to the so called "non-conventional" or "innate" T cells. The best studied examples of these non-conventional T cell lineages are the CD1d specific natural killer T (NKT) cells. These cells are either $CD4^+$ or $CD4^-$ $CD8^-$ (4). NKT cell selection requires

their ligation to glycolipid antigens presented by CD1d on DP cortical thymocytes. Type I NKT cells which are also termed invariant NKT cells (iNKT cells) show a very restricted T cell receptor repertoire. In mice they express an invariant Vα14-Jα18 chain in combination with certain TCR β-chains (Vβ8.2, Vβ7 or Vβ2) (5, 6). Therefore iNKT cells are a population consisting of some clones that express different combinations of these TCRs mentioned above, but still they uniformly react to CD1d tetramers loaded with the glycoshpingolipid antigen α-galactosylseramide, allowing the iNKT cell population to be tracked as a whole. After selection, iNKT cell precursors undergo a series of differentiation steps characterized by the expression of defined surface markers. The most immature iNKT cells are HSA^{high} followed by a HSA^{low} stage, the so called stage 1 where they do not express CD44 or NK1.1. When iNKT cells progress into stage 2, they up regulate CD44, and in a final maturation step they start to express NK1.1 (stage 3) (7). Part of the immature iNKT cells at stage 2 already can leave the thymus and show final maturation in the periphery. However some very mature stage 3 iNKT cells reside in the thymus, the reason for this phenomenon is not clear to date. If mature iNKT cells in the periphery encounter their antigen they are capable to show immediate effector function like the production of IL-4 and IFN-γ. The defined function of iNKT cells in the immune system still needs to be further investigated but there is clear evidence that iNKT cells are able to enhance microbial immunity and tumor rejection, but also promote tolerance and suppress autoimmune diseases (8).

An additional innate T cell lineage develops from a double-positive progenitor that is $CD8^+$ as a mature cell. These non-conventional $CD8^+$ T cells express a TCR specific for non-classical MHC class Ib molecules, like H2-M3 (histocompatibility 2, M region locus 3), Qa-1 (H2-T23) and MR1 (MHC class I related) (4).

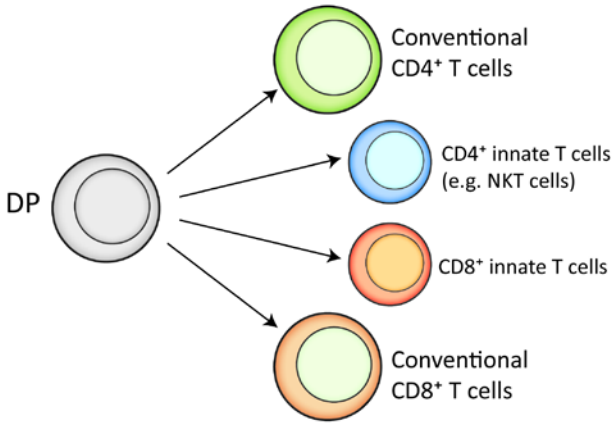

Fig.2 is showing the T cell lineages which develop from the DP progenitor cells in the thymus, like conventional naïve $CD4^+$ and $CD8^+$ T cells, $CD4^+$ innate T cells (e.g. NKT cells) and $CD8^+$ innate T cells.

The two main differences between conventional and non-conventional T cell development are the differences in the TCR repertoire and the dependence on interactions with epithelial cells versus bone marrow derived cells, such as thymocytes. In contrast to conventional naïve T cells, which undergo positive selection following moderate affinity interactions with classical MHC molecules expressed on thymic epithelial cells, non-conventional T cells are selected by TCR interactions with non-classical MHC molecules expressed on thymocytes (4). DP thymocytes provide signals to the selected T cell which are not provided by thymic epithelial cells, therefore contributing to the innate T cell development. Homotypic interactions across DP cell-DP cell contacts generate these second signal mediated by engagement of hemophilic receptors of members of the signaling lymphocytic activation molecule (SLAM) family (9).

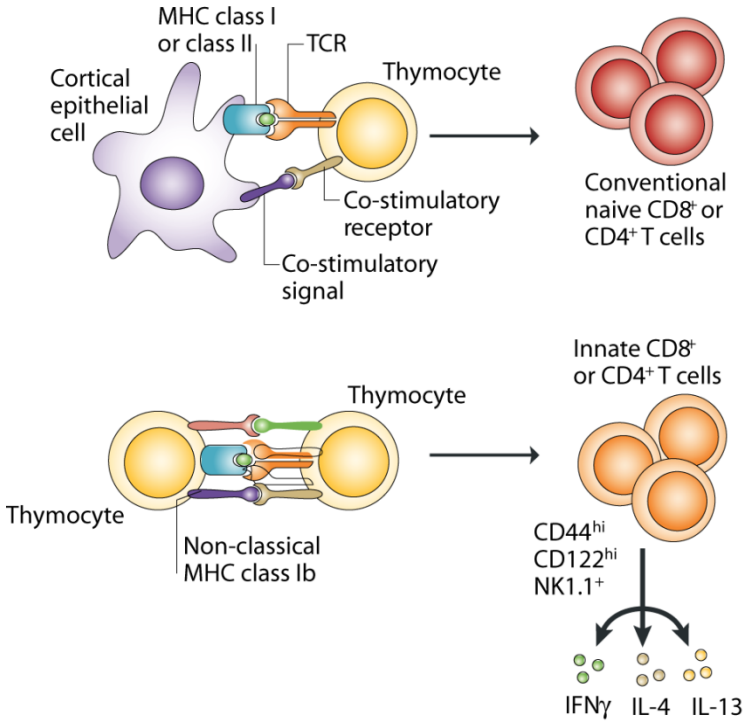

Fig.3 shows the differences between conventional versus non-conventional T cell development (modified from *Berg L. Nature Reviews Immunology 2007*). Conventional T cells are selected in the thymus by thymic epithelial cells on MHC class I or II, while innate T cells are selected via interaction with non classical MHC molecules presented on bone marrow derived thymocytes.

Innate T cells share some characteristics as the expression of defined surface markers like $CD44^{hi}$, $CD122^{hi}$ and some express NK1.1, as well as they all show immediate effector functions (cytokine production) and some are dependent on IL-15 for their survival (e.g. NKT cells). In general it is thought that these innate T cells may serve as an important so called first line of de-

fense against certain bacteria and may therefore build kind of a bridge between the innate and adaptive immunity (10).

Development of the γδ-TCR lineage: During the double-negative stages in the thymus, DN3-stage thymocytes not only rearrange the β-chains, but simultaneously also the γ- and δ-chains. If a complete γδ-TCR is formed before a successful β-chain rearrangement has led to the production of a pre-TCR, the thymocytes already receive a signal through the γδ-TCR. This signal leads to the cessation of the β-chain rearrangement and the cell has been committed to the γδ-TCR lineage. Mature γδ-T cells mainly reside in the gut and strikingly do not seem to require antigen processing and MHC presentation of peptide epitopes although some recognize MHC class IB molecules. Thus γδ-T cells also seem to play a role in bridging the innate and adaptive immune response like the non-conventional αβ-T cells (11).

1.1.2 Transcription factors involved in T cell development

To make choices between T-lineages transcription factors and signaling molecules are used throughout the T cell development to establish T cell identity and sub specializations. These molecules include Notch proteins and their transcriptional effector RBPSuh(CSL);GATA-3; the bHLH factors E2A and HEB and their antagonists Id2 and Id3;Runx1 and Runx3; and also members of the Ikaros family. The individual functions these factors perform are stage-specific, discontinuous and even subject to alternation between activating and inhibitory effects from one stage to the next (for review see(12)).

BTB domain-containing zinc finger proteins

Several members of the BTB (bric-a-brac broad complex) domain-containing family of zinc finger proteins (BTB-ZF) have been described to be important for the development and function of T lymphocytes: MAZR, Th-POK (also named cKrox), Bcl6, BAZF (Bcl6b), ROG (PLZP /FAZF) and PLZF. MAZR was shown to be a repressor of CD8 expression in early thymocyte development (13), while a more recent study in our laboratory could show a role for MAZR in CD4/CD8 cell fate decisions during thymocyte development (Shinya Sakaguchi, unpublished data). cKrox also known as Th-POK is an important master regulator of helper lineage commitment (14). In contrast Bcl6 seems to play a more important role in regulating T cell function rather than T cell development. Studies have implicated Bcl-6 in the repression of GATA-3, therefore negatively regulating the development of TH2 cells (15). Another study could show a function for Bcl-6 in

the generation of $CD8^+$ memory T cells, especially for the development of the so called central memory subset (16). BAZF/Bcl6b a quite close relative to Bcl-6 seems to be important in naïve $CD4^+$ T cells. BAZF/Bcl6b deficient $CD4^+$ T cells show impaired proliferation and IL-2 production upon T cell receptor triggering (17). However this was not observed by another group which independently generated Bcl6b knock-out mice. This additional studies rather could show a function for Bcl6b in the generation of a secondary response of $CD8^+$ memory T cells (18). Studies with ROG/PLZP deficient mice implicated this factor to be a negative regulator of T cell proliferation and IL-2 production upon engagement of the T cell receptor (19). This proteins contain an N-terminal BTB-POZ domain which is necessary for their interaction with e.g. HDAC1 (histone deacetylase 1), mSin3a (Swi independent 3A), SMRT (silencing mediator of retinoid and thyroid hormone receptors) and NCoR (nuclear Corepressor), thereby mediating transcriptional repression. The C-terminal Zinc-finger domains are required for DNA binding (for review see (20)).

PLZF (Promyelocytic Leukemia Zinc Finger)

Another member of the BTB-ZF family of transcription factors PLZF became famous as a fusion protein with the retinoic acid receptor α (RARα) protein. This PLZF-RARα fusion protein leads to acute promyelocytic leukemia (APL) (for review see(21)). Additional studies could show the importance of PLZF for limb development and spermatogenesis (22-25). Two recent studies implicated PLZF in NKT cell development and function. These studies which were performed using PLZF deficient mice have shown that in the absence of PLZF very few NKT cells develop and that the remaining NKT cells show impaired effector functions and do not acquire the phenotype and innate-like characteristics usually associated with iNKT cells (26, 27). An additional study could show that enforced expression of PLZF can drive effector functions on $CD4^+$ T cells (27).

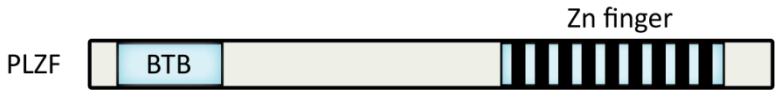

Fig.4 shows a schematic map of the BTB-ZF protein PLZF. The protein consists of an N-terminal BTB domain and 9 C-terminal Zinc finger domains. The protein domain structure is commonly shared within the BTB-ZF family of transcription factors.

1.1.3 T cell homeostasis

Mature single-positive thymocytes emigrate from the thymus to the peripheral organs of the immune system- the lymph nodes, spleen and the mucosal lymphoid tissues. In the absence of an infection the size and composition of the peripheral T cell pool has to be tightly controlled. Peripheral T cells should be kept constant in numbers and should contain diverse but potentially functional T cell receptors. This regulatory process is known as T cell homeostasis.

It has been shown that the cytokine interleukin-7 (IL-7) plays an important role in the survival of peripheral T cells, thus a limiting amount of this cytokine keeps the T cell pool constant in size. Transfers of naïve T cells into mice which do not contain a T cell pool (e.g. RAG knock-out mice, a so called “empty host”) have shown that these transferred naïve cells proliferate and acquire a memory phenotype. This could be explained by the plethora of survival factors in this “empty organs” and a reduced competition for these factors. Furthermore there seems to be a requirement for interactions with self-peptide/self MHC-complexes, which leads mature naïve T cells to undergo infrequent cell divisions. This slight increase in T cell numbers is balanced by loss of T cells most likely among daughter cells of the dividing naïve cells (28).

1.1.4 T cell receptor signaling

Signal transduction by the T cell receptor is linking antigen recognition with a functional response of the T cell. The recognition of antigen initiates a sequence of biochemical signals in T cells that result in proliferation and lead to the expression of various genes. Not only the signal through the TCR (signal 1) which is mediated by the binding of the foreign-peptide/self MHC complex, is important to fully activate a naïve T cell. Effective activation also requires a co-stimulatory signal (signal 2) transmitted through the CD28 coreceptor, to be delivered by the same antigen presenting cell (APC) which leads to IL-2 production and increased survival of the T cell. If a T cell recognizes antigen in the absence of co-stimulatory molecules, it receives signal 1 alone and is inactivated to prevent potentially self reactivity and allows self antigens expressed on tissue cells to induce tolerance in the peripheral T cell population. Once the cells have differentiated into effector cells, any encounter of the antigen which the cells are responding to, triggers their effector actions without the need for co-stimulatory signals.

Tec kinases and T cell receptor signaling

The Tec family tyrosine kinases are known to be important mediators of antigen receptor signaling in lymphocytes. Three members are expressed in T cells and known to be activated in response to T cell receptor signaling: Itk, Tec and Rlk (29).

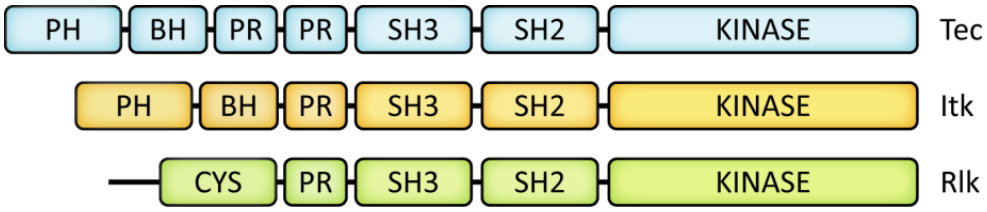

Fig.5 is showing the three members of the Tec family kinases which are expressed in T lymphocytes: Tec, Ikt and Rlk. Abbreviations: PH, Pleckstrin homology domain; BH, Btk homology motiv; PR, proline rich domain; SH3, Src homology domain 3; SH2, Src homology domain 2; KINASE, Kinase domain; CYS, cystein string motiv;

Activation of the TCR receptor by antigen binding results in the activation of src family kinases Lck and Fyn and the phosphorylation of ITAM motifs on the CD3 subunits. Zap-70 is bound by these immunoreceptor tyrosine-based activation motifs (ITAM) and activated by phosphorylation. Activated Zap-70 phosphorylates the docking protein, linker for activation of T cells (LAT). Several molecules bind to LAT including PLCγ1 and a trimeric complex composed of SLP-76, Vav1 and ITK. Tec kinases are recruited to the plasma membrane through interactions of their pleckstrin homology (PH) domain with $PI_{(3,4,5)}P_3$, the product of the phosphoinositid-3 kinase (PI3K). Therefore Tec kinases can be negatively regulated by the phosphatases PTEN and SHIP, which can reduce the levels of $PI_{(3,4,5)}P_3$ in the plasma membrane, leading to reduced recruitment of Tec kinases to the site of action. Rlk which does not contain a PH domain is recruited to the membrane by palmitoylation of the cysteine string motif and is therefore independent of PI3K. Two steps of tyrosine phosphorylations are needed for an activation of the Tec kinases, the trans-phosphorylation which is performed by the Src kinases, followed by an auto-phosphorylation (29). In addition interactions with other signaling molecules, a so called signaling complex, integrates downstream signaling events to PLCγ and the guanine nucleotide exchange factor Vav1.

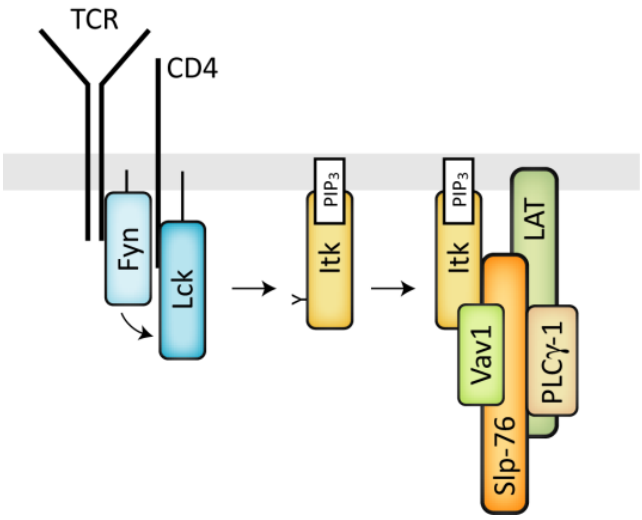

Fig.6 shows an illustration of the early events in T cell receptor signaling and the formation of the signalosome. Upon T cell receptor triggering Fyn activates Lck which in turn phosphorylates Itk. The Tec kinase Itk is recruited to the plasma membrane by PI3K which is responsible for the production of $PI_{(3,4,5)}P_3$. After an auto-phosphorylation Itk becomes part of the signaling complex, the signalosome, leading to downstream events necessary for T cell activation.

Itk seems to play the most important role in T lymphocytes among the other Tec family kinases Tec and Rlk. Studies with *Itk-/-* mice have shown the importance of Itk in T cell development. These mice have reduced numbers of mature T cells, decreased ratios of CD4/CD8 single-positive cells and defects in thymic positive selection (30). More recent studies have shown the importance of Itk in the development of conventional naïve $CD8^+$ T cells, since Itk deficient mice are almost devoid of conventional $CD8^+$ T cells. The remaining $CD8^+$ T cells in these mice show innate-like characteristics and are selected on non-classical MHC class Ib molecules presented on bone marrow derived cells in the thymus (31-33).

Mature Itk deficient T cells show impaired responses to TCR stimulation including proliferation, Interleukin-2 (IL-2) production and expression of effector cytokines (34, 35). Furthermore Itk deficient mice show defects in peripheral T cell activation and in the polarization towards a TH2 helper phenotype (36).

Crosstalk between Itk and other T cell receptor signaling molecules: As already mentioned above the Tec kinase Itk is known to be an important molecule of the signaling complex formed upon TCR engagement, and therefore necessary for an efficient T cell activation. Vav1 a Dbl homology domain containing GDP/GTP guanine exchange factor that activates members of the Rho GTPases family (for reviews see (37, 38)) has also been shown to be necessary for a proper synapse formation and efficient T cell activation. Furthermore Vav1 can interact with Itk (39), and

both Vav1 and Itk are phosphorylated upon TCR stimulation (40, 41). It has been shown that the recruitment of Vav1 to the immunological synapse may be dependent on Itk (39, 42). More recent data suggest that this recruitment could be mediated through a kinase-independent function of Itk (39, 42, 43).

Another TCR signaling protein, the E3 ubiquitin ligase Cbl-b, which modulates activation thresholds in T cells and prevents the development of autoimmunity (44-46), negatively regulates Vav1-mediated signaling, acting via the CD28 co-stimulatory pathway (47). The similarities between the biochemical and cellular defects of Itk and Vav1 deficient T cells also leads to the suggestion of a potential crosstalk between Itk and Cbl-b and not only between Itk and Vav1.

1.1.5 Effector functions of T cells

At the end of the proliferative phase of a T cell response induced by TCR engagement plus co-stimulatory signals, activated T cells differentiate into effector T cells. This differentiated T cells can synthesize the effector molecules which are required for their helper or cytotoxic functions. Depending on the surrounding cytokine milieu, especially for $CD4^+$ helper T cells, this maturation can result in the development of various functionally different T cell subsets. In contrast $CD8^+$ T cells always differentiate into cytotoxic T cells which kill their (mainly virus infected) target cells by expression of perforin, granzymes or fas-ligand.

$CD4^+$ effector T cell subsets

TH17 and regulatory T cells: In the absence of an infection dendritic cells rather produce TGF-β than IL-6 leading to an induction of FOXP3 expression if a $CD4^+$ T cell encounters antigen, resulting in a regulatory phenotype. Regulatory T cells (T_{regs}) are involved in closing down immune responses after successful clearance of the antigen, and also in keeping in check responses that may potentially be against self. However in an early phase of an infection $CD4^+$ T cell differentiation shifts from a regulatory to a TH17 phenotype, due to increased access of IL-6 and lower amounts of TGF-β. The cytokine IL-6 leads to the expression of RORγt by the activated T cell and causes the production of IL-17 and IL-17f, specifically secreted by the TH17 cell subset. TH17 cells can be maintained by IL-23 and are known to be important mediators in an inflammatory response but are also implicated in some autoimmune diseases.

TH1 and TH2 effector cells: Pathogens like intracellular bacteria or viruses activate dendritic cells to produce interleukin-12 (IL-12) and Natural killer cells (NK cells) to produce IFNγ. This causes proliferating $CD4^+$ T cells to polarize into the TH1 subset, by activation of the JAK-STAT signaling pathway. IFNγ activates STAT1 which in turn induces the expression of T-bet. This transcription factor binds to the locus of the IFNγ gene and turns on the expression of IFNγ. From then on these cells are committed into the TH1 subset and mainly produce IFNγ but also IL-2 and TNF-α. These TH1 helper T cell cytokines are necessary to activate macrophages, therefore contributing to the clearance of intracellular pathogens.

IL-4 can be produced by various cells (e.g. NKT cells) and is made upon infection with parasitic worms and other pathogens acts on proliferating $CD4^+$ T cells to cause them into a TH2 cell. The access of IL-4 leads to activation of STAT6 which promotes the expression of the transcription factor GATA-3 in the activated T cell. GATA-3 is an activator of cytokines produced by TH2 cells such as IL-4, but it also activates its own expression, therefore not only induces but also maintains the differentiation of the TH2 subset. TH2 cytokines mainly provide help to B cells by activating them to become antibody secreting plasma cells (48).

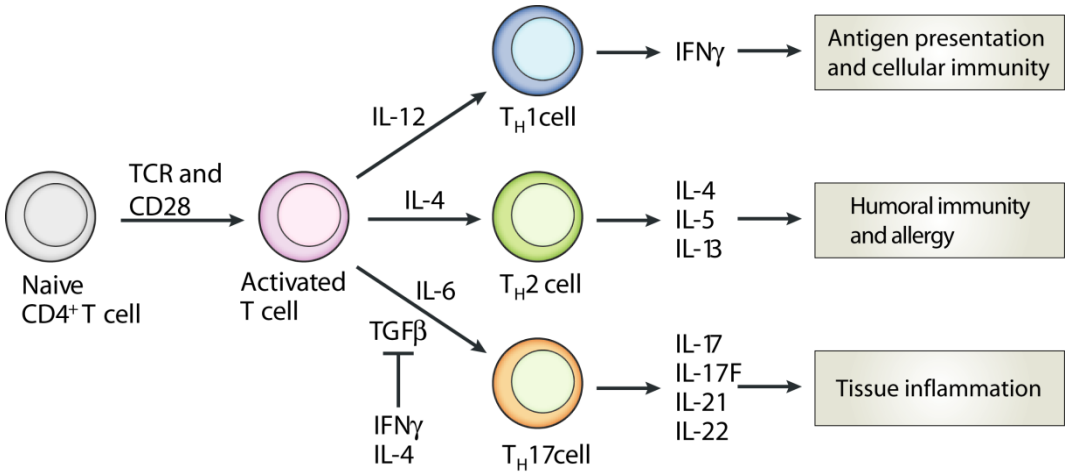

Fig.7 (modified from *Dong C., Nature Immunology Reviews 2008*) is showing a simplified scheme of the differentiation pathways of activated $CD4^+$ T cells into different effector T cell subsets.

Each of the $CD4^+$ T cell subsets produces cytokines which negatively regulate the development or effector activity of the other subsets, thus allowing the upcoming T cell subset to dominate an immune response.

Memory T cells and memory-phenotype T cell populations

Peripheral $CD4^+$ and $CD8^+$ T cell subsets have been traditionally divided into naïve $CD44^{lo}CD62L^+$ and memory $CD44^{hi}CD62L^-$ populations, while the surface expression phenotype of the latter population also resembles recently activated T cells (49, 50). In the last years it became clear that the memory T cell subset is not a population consisting only of true antigen-specific memory cells that developed in response to a foreign antigen. Rather, the memory population contains a variety of different T lymphocyte subsets, some may have acquired their memory phenotype through homeostatic proliferation, while others show immediate effector function and may play a role in the front-line defense against certain bacterial infections. These additional cells were described as memory-phenotype (MP) T lymphocytes and some subsets of the MP population also as innate T cells (4, 51).

Immunological memory enables the immune system to respond more rapidly and efficiently to pathogens that have been encountered already previously. After an immunization the number of T cells reactive to the given antigen increases heavily as effector T cells but is going down again after the clearance of the pathogen. Nevertheless a small part of cells persists after an immune response, the so called memory T cells. These memory T cells are long-lived cells with a defined expression of cell surface molecules, particular responses to stimuli and the expression of certain genes controlling cell survival. Memory T cells for example express high levels of the adhesion molecule CD44 and the survival factor Bcl-2. Two types of memory T cells can derive from a primary immune response.

Effector memory cells express the chemokine receptors CCR3 and CCR5, high levels of CD44, but are $CD62L^-$. After re-stimulation these cells rapidly mature into effector cells and are capable to secrete high levels of IFN-γ, IL-4 and IL-5.

Central memory cells express CCR7 and show a $CD44^{hi}CD62L^+$ surface marker expression. They remain in the peripheral lymphoid tissues after re-stimulation and take longer than effector memory cells to develop into effector cells, therefore do not secrete that high levels of cytokines.

1.2 References

1. Germain, R.N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol* 2:309-322.
2. Godfrey, D.I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol Today* 14:547-553.
3. von Boehmer, H., I. Aifantis, F. Gounari, O. Azogui, L. Haughn, I. Apostolou, E. Jaeckel, F. Grassi, and L. Klein. 2003. Thymic selection revisited: how essential is it? *Immunol Rev* 191:62-78.
4. Berg, L.J. 2007. Signalling through TEC kinases regulates conventional versus innate CD8(+) T-cell development. *Nat Rev Immunol* 7:479-485.
5. MacDonald, H.R. 2002. Development and selection of NKT cells. *Curr Opin Immunol* 14:250-254.
6. Berzins, S.P., A.P. Uldrich, D.G. Pellicci, F. McNab, Y. Hayakawa, M.J. Smyth, and D.I. Godfrey. 2004. Parallels and distinctions between T and NKT cell development in the thymus. *Immunol Cell Biol* 82:269-275.
7. Godfrey, D.I., and S.P. Berzins. 2007. Control points in NKT-cell development. *Nat Rev Immunol* 7:505-518.
8. Tupin, E., Y. Kinjo, and M. Kronenberg. 2007. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol* 5:405-417.
9. Griewank, K., C. Borowski, S. Rietdijk, N. Wang, A. Julien, D.G. Wei, A.A. Mamchak, C. Terhorst, and A. Bendelac. 2007. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* 27:751-762.
10. Taniguchi, M., K. Seino, and T. Nakayama. 2003. The NKT cell system: bridging innate and acquired immunity. *Nat Immunol* 4:1164-1165.
11. Hayday, A.C., and D.J. Pennington. 2007. Key factors in the organized chaos of early T cell development. *Nat Immunol* 8:137-144.
12. Rothenberg, E.V., and T. Taghon. 2005. Molecular genetics of T cell development. *Annu Rev Immunol* 23:601-649.
13. Bilic, I., C. Koesters, B. Unger, M. Sekimata, A. Hertweck, R. Maschek, C.B. Wilson, and W. Ellmeier. 2006. Negative regulation of CD8 expression via Cd8 enhancer-mediated recruitment of the zinc finger protein MAZR. *Nat Immunol* 7:392-400.

14. He, X., X. He, V.P. Dave, Y. Zhang, X. Hua, E. Nicolas, W. Xu, B.A. Roe, and D.J. Kappes. 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature* 433:826-833.

15. Dent, A.L., J. Hu-Li, W.E. Paul, and L.M. Staudt. 1998. T helper type 2 inflammatory disease in the absence of interleukin 4 and transcription factor STAT6. *Proc Natl Acad Sci U S A* 95:13823-13828.

16. Ichii, H., A. Sakamoto, M. Hatano, S. Okada, H. Toyama, S. Taki, M. Arima, Y. Kuroda, and T. Tokuhisa. 2002. Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells. *Nat Immunol* 3:558-563.

17. Takamori, M., M. Hatano, M. Arima, A. Sakamoto, L. Fujimura, T. Hartatik, T. Kuriyama, and T. Tokuhisa. 2004. BAZF is required for activation of naive CD4 T cells by TCR triggering. *Int Immunol* 16:1439-1449.

18. Manders, P.M., P.J. Hunter, A.I. Telaranta, J.M. Carr, J.L. Marshall, M. Carrasco, Y. Murakami, M.J. Palmowski, V. Cerundolo, S.M. Kaech, R. Ahmed, and D.T. Fearon. 2005. BCL6b mediates the enhanced magnitude of the secondary response of memory CD8+ T lymphocytes. *Proc Natl Acad Sci U S A* 102:7418-7425.

19. Kang, B.Y., S.C. Miaw, and I.C. Ho. 2005. ROG negatively regulates T-cell activation but is dispensable for Th-cell differentiation. *Mol Cell Biol* 25:554-562.

20. Bilic, I., and W. Ellmeier. 2007. The role of BTB domain-containing zinc finger proteins in T cell development and function. *Immunol Lett* 108:1-9.

21. Kelly, K.F., and J.M. Daniel. 2006. POZ for effect--POZ-ZF transcription factors in cancer and development. *Trends Cell Biol* 16:578-587.

22. Costoya, J.A., R.M. Hobbs, M. Barna, G. Cattoretti, K. Manova, M. Sukhwani, K.E. Orwig, D.J. Wolgemuth, and P.P. Pandolfi. 2004. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat Genet* 36:653-659.

23. Barna, M., P.P. Pandolfi, and L. Niswander. 2005. Gli3 and Plzf cooperate in proximal limb patterning at early stages of limb development. *Nature* 436:277-281.

24. Barna, M., T. Merghoub, J.A. Costoya, D. Ruggero, M. Branford, A. Bergia, B. Samori, and P.P. Pandolfi. 2002. Plzf mediates transcriptional repression of HoxD gene expression through chromatin remodeling. *Dev Cell* 3:499-510.

25. Barna, M., N. Hawe, L. Niswander, and P.P. Pandolfi. 2000. Plzf regulates limb and axial skeletal patterning. *Nat Genet* 25:166-172.

26. Kovalovsky, D., O.U. Uche, S. Eladad, R.M. Hobbs, W. Yi, E. Alonzo, K. Chua, M. Eidson, H.J. Kim, J.S. Im, P.P. Pandolfi, and D.B. Sant'Angelo. 2008. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol* 9:1055-1064.

27. Savage, A.K., M.G. Constantinides, J. Han, D. Picard, E. Martin, B. Li, O. Lantz, and A. Bendelac. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29:391-403.

28. Surh, C.D., and J. Sprent. 2005. Regulation of mature T cell homeostasis. *Semin Immunol* 17:183-191.

29. Berg, L.J., L.D. Finkelstein, J.A. Lucas, and P.L. Schwartzberg. 2005. Tec family kinases in T lymphocyte development and function. *Annu Rev Immunol* 23:549-600.

30. Lucas, J.A., A.T. Miller, L.O. Atherly, and L.J. Berg. 2003. The role of Tec family kinases in T cell development and function. *Immunol Rev* 191:119-138.

31. Broussard, C., C. Fleischacker, R. Horai, M. Chetana, A.M. Venegas, L.L. Sharp, S.M. Hedrick, B.J. Fowlkes, and P.L. Schwartzberg. 2006. Altered development of CD8+ T cell lineages in mice deficient for the Tec kinases Itk and Rlk. *Immunity* 25:93-104.

32. Atherly, L.O., J.A. Lucas, M. Felices, C.C. Yin, S.L. Reiner, and L.J. Berg. 2006. The Tec family tyrosine kinases Itk and Rlk regulate the development of conventional CD8+ T cells. *Immunity* 25:79-91.

33. Hu, J., N. Sahu, E. Walsh, and A. August. 2007. Memory phenotype CD8+ T cells with innate function selectively develop in the absence of active Itk. *Eur J Immunol* 37:2892-2899.

34. Liao, X.C., and D.R. Littman. 1995. Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. *Immunity* 3:757-769.

35. Liao, X.C., S. Fournier, N. Killeen, A. Weiss, J.P. Allison, and D.R. Littman. 1997. Itk negatively regulates induction of T cell proliferation by CD28 costimulation. *J Exp Med* 186:221-228.

36. Schaeffer, E.M., G.S. Yap, C.M. Lewis, M.J. Czar, D.W. McVicar, A.W. Cheever, A. Sher, and P.L. Schwartzberg. 2001. Mutation of Tec family kinases alters T helper cell differentiation. *Nat Immunol* 2:1183-1188.

37. Cantrell, D. 2003. Commentary: Vav-1 and T cells. *Eur J Immunol* 33:1070-1072.

38. Tybulewicz, V.L. 2005. Vav-family proteins in T-cell signalling. *Curr Opin Immunol* 17:267-274.

39. Dombroski, D., R.A. Houghtling, C.M. Labno, P. Precht, A. Takesono, N.J. Caplen, D.D. Billadeau, R.L. Wange, J.K. Burkhardt, and P.L. Schwartzberg. 2005. Kinase-independent functions for Itk in TCR-induced regulation of Vav and the actin cytoskeleton. *J Immunol* 174:1385-1392.

40. Crespo, P., K.E. Schuebel, A.A. Ostrom, J.S. Gutkind, and X.R. Bustelo. 1997. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature* 385:169-172.

41. Heyeck, S.D., H.M. Wilcox, S.C. Bunnell, and L.J. Berg. 1997. Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity. *J Biol Chem* 272:25401-25408.

42. Labno, C.M., C.M. Lewis, D. You, D.W. Leung, A. Takesono, N. Kamberos, A. Seth, L.D. Finkelstein, M.K. Rosen, P.L. Schwartzberg, and J.K. Burkhardt. 2003. Itk functions to control actin polymerization at the immune synapse through localized activation of Cdc42 and WASP. *Curr Biol* 13:1619-1624.

43. Grasis, J.A., C.D. Browne, and C.D. Tsoukas. 2003. Inducible T cell tyrosine kinase regulates actin-dependent cytoskeletal events induced by the T cell antigen receptor. *J Immunol* 170:3971-3976.

44. Bachmaier, K., C. Krawczyk, I. Kozieradzki, Y.Y. Kong, T. Sasaki, A. Oliveira-dos-Santos, S. Mariathasan, D. Bouchard, A. Wakeham, A. Itie, J. Le, P.S. Ohashi, I. Sarosi, H. Nishina, S. Lipkowitz, and J.M. Penninger. 2000. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* 403:211-216.

45. Chiang, Y.J., H.K. Kole, K. Brown, M. Naramura, S. Fukuhara, R.J. Hu, I.K. Jang, J.S. Gutkind, E. Shevach, and H. Gu. 2000. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* 403:216-220.

46. Loeser, S., and J.M. Penninger. 2007. Regulation of peripheral T cell tolerance by the E3 ubiquitin ligase Cbl-b. *Semin Immunol* 19:206-214.

47. Krawczyk, C., K. Bachmaier, T. Sasaki, R.G. Jones, S.B. Snapper, D. Bouchard, I. Kozieradzki, P.S. Ohashi, F.W. Alt, and J.M. Penninger. 2000. Cbl-b is a negative regulator of receptor clustering and raft aggregation in T cells. *Immunity* 13:463-473.

48. Dong, C. 2008. TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat Rev Immunol* 8:337-348.

49. Swain, S.L., J.N. Agrewala, D.M. Brown, D.M. Jelley-Gibbs, S. Golech, G. Huston, S.C. Jones, C. Kamperschroer, W.H. Lee, K.K. McKinstry, E. Román, T. Strutt, and N.P. Weng.

2006. CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. *Immunol Rev* 211:8-22.

50. Prlic, M., M.A. Williams, and M.J. Bevan. 2007. Requirements for CD8 T-cell priming, memory generation and maintenance. *Curr Opin Immunol* 19:315-319.

51. Surh, C.D., O. Boyman, J.F. Purton, and J. Sprent. 2006. Homeostasis of memory T cells. *Immunol Rev* 211:154-163.

2

AIM OF THE THESIS

The Tec kinase Itk is known to be an important mediator of T cell receptor signaling. Mice deficient for this tyrosine kinase show a similar phenotype as mice deficient for the guanine nucleotide exchange factor Vav1 which also plays an important role upon T cell receptor triggering. This fact, in combination with several additional studies (for further details see part 3.1.2) and the observation that this two proteins interact, provided evidence that there are functional crosstalks between Itk and Vav1 during T cell receptor signaling. However it was still unclear whether the functions of Vav1 and Itk are partially redundant and whether there are crosstalks between these molecules not only during T cell receptor signaling in mature T cells but also during T cell development.

In order to investigate whether these two molecules indeed function in the same genetic pathways and whether there is functional redundancy between Vav1 and Itk during T cell activation, we generated Vav1/Itk double deficient mice. Since Vav1 and Itk both interact with members of the Cbl family of E3 ubiquitin ligases, and signaling defects in *Vav1*$^{-/-}$ T cells are rescued upon deletion of Cbl-b, we further wanted to investigate the relation between Itk and Cbl-b by generating Itk/Cbl-b double-deficient mice.

In addition we wanted to determine a potential role of the transcription factor PLZF (promyelocytic leukemia zinc finger) in the T cell lineage since this protein was identified in an Affymetrix microarray to be up-regulated in Itk deficient T cells. *Itk–/–* mice have a T cell memory phenotype (MP), and we could show that the increase in the expression of PLZF in *Itk-/-* T cells was due to a higher percentage of MP T cells. Furthermore we observed that wildtype memory phenotype T cells also express high levels of PLZF. These data indicated a correlation between PLZF and memory phenotype T cells. To gain more insight into a potential role of PLZF during T cell development and memory phenotype T cell function, we generated T cell specific PLZF transgenic mice.

3

RESULTS

During my PhD I could publish two first author papers, which are included below. I planned and performed the experiments and contributed to the design of the research and the writing of the manuscripts. The manuscript of a third study were I contributed is already submitted. Furthermore I was writing a chapter of a review about Tec kinases in inflammatory processes.

Impaired T cell development in the absence of Vav1 and Itk

Raberger, J., Boucheron , N. , Sakaguchi, S., Penninger, J.M. and Ellmeier , W. Impaired T cell development in the absence of Vav1 and Itk. *Eur J Immunol. 2008 Nov 13*

The transcriptional regulator PLZF induces the development of CD44 high memory phenotype T cells

Raberger, J, Schebesta, A., Sakaguchi, S., Boucheron, N., Blomberg , K.M. , Berglöf , A. , Kolbe , T., Smith , C.I.E. , Rülicke , T. and Ellmeier, W. The Transcriptional Regulator PLZF Induces the Development of CD44-High Memory-Phenotype T Cells. *Proc Natl Acad Sci U S A. 2008 Nov 18;105 (46):17919-17924. Epub 2008 Nov 12*

The protein tyrosine kinase Tec regulates IL-17-producing memory-like CD4$^+$ T cells

Nicole Boucheron[1], Benjamin Vigl[1,5,*], Julia Raberger[1,*], Andrew Croxford[2,*], Alexandra Schebesta[1,*], Uwe Schmidt[1,6,*], Jan Bauer[3], Rashmi Bankoti[4], Michelle M. Epstein[4], Hans Lassmann[3], Ari Waisman[2] and Wilfried Ellmeier[1]

The role of Tec family kinases in inflammatory processes

Melcher M., Raberger J., Schmidt U., Unger B. and Ellmeier W. Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry, 2007

3.1 Impaired T cell development in the absence of Vav1 and Itk

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European Journal of Immunology

Impaired T-cell development in the absence of Vav1 and Itk

Julia Raberger[1], Nicole Boucheron[1], Shinya Sakaguchi[1], Josef M. Penninger[2] and Wilfried Ellmeier[1]

[1] Division of Immunobiology, Institute of Immunology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, Vienna, Austria
[2] Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria

Vav1 and the Tec family kinase Itk act in similar T-cell activation pathways. Both molecules interact with members of the Cbl family of E3 ubiquitin ligases, and signaling defects in *Vav1*$^{-/-}$ T cells are rescued upon deletion of Cbl-b. In this study we investigate the relation between Itk and Cbl-b or Vav1 by generating Itk/Cbl-b and Itk/Vav1 double-deficient mice. Deletion of Cbl-b in *Itk*$^{-/-}$ CD4^{+} T cells restored proliferation and partially IL-2 production, and also led to a variable rescue of IL-4 production. Thus, Itk and Vav1 act mechanistically similarly in peripheral T cells, since the defects in *Itk*$^{-/-}$ T cells, as in *Vav1*$^{-/-}$ T cells, are rescued if cells are released from the negative regulation mediated by Cbl-b. In addition, only few peripheral CD4^{+} and CD8^{+} T cells were present in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice due to severely impaired thymocyte differentiation. *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocyte numbers were strongly reduced compared with WT, *Itk*$^{-/-}$ or *Vav1*$^{-/-}$ mice, and double-positive thymocytes displayed increased cell death and impaired positive selection. Therefore, our data also reveal that the combined activity of Vav1 and Itk is required for proper T-cell development and the generation of the peripheral T-cell pool.

Key words: Kinases · Knockout mice · Signal transduction · T cells


Supporting Information available online

Introduction

TCR/CD28 triggering by APC induces a signaling cascade that leads to the activation of signaling molecules, the reorganization of the actin cytoskeleton and the formation of the so-called immunological synapse at the T-cell/APC interphase. Together this leads to the activation of downstream signaling components and activation of transcription factors resulting in the induction of IL-2 expression, proliferation of the T cells and the generation of activated effector T-cell populations, which is important for the development of a proper adaptive immune response [1, 2].

Molecules such as Vav1 and wasp (for review see [3]), and also the Tec family kinase Itk, [4] have been shown to be necessary for proper synapse formation and for efficient T-cell activation. Vav1 is a Dbl homology domain containing GDP/GTP guanine exchange factor that activates members of the Rho GTPases family (for reviews see [5, 6]). Genetic studies have shown impaired development of *Vav1*$^{-/-}$ thymocytes indicated by reduced numbers of total thymocytes due to a partial block at the double-negative (DN) stage. Furthermore, the double-positive (DP) subset of *Vav1*$^{-/-}$ thymocytes shows

Correspondence: Dr. Wilfried Ellmeier
e-mail: wilfried.ellmeier@meduniwien.ac.at

impaired positive selection leading to reduced numbers of peripheral T cells, and during T-cell activation Vav1 is required for efficient TCR-mediated T-cell proliferation and cytokine production [7–11]. Vav1 controls integrin clustering and TCR stimulation-induced cytoskeletal reorganization required for the formation of the immunological synapse, since $Vav1^{-/-}$ T cells show impaired receptor clustering upon activation [11]. The E3 ubiquitin ligase Cbl-b, which modulates activation thresholds in T cells and prevents the development of autoimmunity [12–14], negatively regulates Vav1-mediated receptor clustering, since the defect in $Vav1^{-/-}$T cells is relieved in $Vav1^{-/-}Cbl\text{-}b^{-/-}$ double-deficient T cells [15]. Remarkably, mice deficient for the protein tyrosine kinase Itk show a T-cell phenotype similar to that of $Vav1^{-/-}$ mice [16–18]. In addition, $Itk^{-/-}$ $CD4^+$ T cells are not efficiently polarized toward the Th2 lineage [19, 20]. Vav1 interacts with Itk [21]; both Vav1 and Itk are phosphorylated upon TCR stimulation [22, 23], and both can interact with members of the Cbl gene family [24–26]. Furthermore, the similar phenotype of $Itk^{-/-}$ and $Vav1^{-/-}$ T cells suggested that Itk and Vav1 may work in similar signaling pathways or may (partially) fulfill similar function (for review see [27, 28]). $Vav1^{-/-}$ T cells display impaired activation of Itk upon TCR stimulation; however, this effect might be indirectly due to impaired activation of PI3K in the absence of Vav1 [29]. Furthermore, it has been shown that the recruitment of Vav1 to the immunological synapse may be dependent on Itk [4, 21]. This could be regulated *via* Itk-dependent phosphorylation of linker of activated T-cells (LAT) or SH2 domain containing leukocyte protein of 76kDa (SLP76) adaptor proteins, which would generate docking sites for Vav1 (src homology 2 (SH2) domain-mediated), although Vav1 phosphorylation is normal in $Itk^{-/-}$ T cells [30]. More recent data suggest that this recruitment could be mediated through a kinase-independent function of Itk [4, 21, 31]. Since Itk can interact with Vav1, Itk may be required for the recruitment of Vav1 to SLP76 [21]. Together, these data clearly suggest a functional crosstalk between Vav1 and Itk during T-cell signaling. However, it is still unclear whether the functions of Vav1 and Itk are partially redundant (*i.e.* indicating a scaffolding function for Itk) and whether there are crosstalks between these molecules during T-cell development.

In this study we further investigated potential interdependences between Itk and Cbl-b or Vav1. We investigated whether there are mechanistic similarities in the signaling defects of $Vav1^{-/-}$ and $Itk^{-/-}$ T cells. Itk/Cbl-b double-deficient mice were generated to test whether TCR signaling defects in peripheral $Itk^{-/-}$ T cells are rescued in the absence of Cbl-b, as observed for $Vav1^{-/-}$ T cells [15]. The analysis of $Itk^{-/-}Cbl\text{-}b^{-/-}$ $CD4^+$ T cells indicated, in agreement with another study [27], restored proliferation and at least partially rescued IL-2 production compared with $Itk^{-/-}$ $CD4^+$ T cells. Furthermore, $Itk^{-/-}Cbl\text{-}b^{-/-}$ $CD4^+$ T cells displayed increased Th2 polarization and IL-4 production compared with $Itk^{-/-}$ $CD4^+$ T cells, indicating that deletion of Cbl-b can also bypass the requirement of Itk for the efficient generation of IL-4-producing Th2 cells. In addition, by generating Vav1/Itk double-deficient mice, we wanted to genetically determine whether Itk and Vav1 are indeed functioning in the same genetic pathways and whether there is functional redundancy between Vav1 and Itk during T-cell activation. Unexpectedly, $Vav1^{-/-}Itk^{-/-}$ mice showed severely reduced numbers of peripheral T cells due to impaired thymocyte differentiation. $Vav1^{-/-}Itk^{-/-}$ thymocyte numbers were strongly reduced, the CD4/CD8 developmental profile was dramatically altered and the DP subsets displayed increased cell death compared with WT, $Itk^{-/-}$ or $Vav1^{-/-}$ mice. In addition, mature $CD3^{hi}$ CD4 and CD8 single-positive (SP) thymocytes were absent in $Vav1^{-/-}$ $Itk^{-/-}$ thymi, indicating a block in positive selection. Thus, our data reveal additional similarities between the functional defects of $Vav1^{-/-}$ and $Itk^{-/-}$ T cells, and they show an essential requirement for the combined activity of Vav1 and Itk during T-cell development.

Results

Cbl-b deficiency can rescue T-cell defects of $Itk^{-/-}$ mice

Since it has been shown that Cbl-b deficiency restores signaling in Vav1-deficient T cells [15], we tested whether TCR-signaling defects in peripheral $Itk^{-/-}$ T cells are rescued as well in the absence of Cbl-b. Therefore, $Itk^{-/-}Cbl\text{-}b^{-/-}$ mice were generated. $Itk^{-/-}$ mice have reduced numbers of peripheral T cells [16], and some $CD4^+$ T cells and the majority of $CD8^+$ T cells have an activated/memory-like phenotype [32–35]. Similar numbers of T cells were present in $Itk^{-/-}$ $Cbl\text{-}b^{-/-}$ mice compared with $Itk^{-/-}$ mice, and $Itk^{-/-}$ and $Itk^{-/-}Cbl\text{-}b^{-/-}$ T cells had a similar activated/memory-like phenotype (data not shown), indicating that the homeostasis defect of Itk-deficient mice is not rescued by deletion of Cbl-b.

To investigate whether Cbl-b rescues the proliferation defect of $Itk^{-/-}$ T cells, peripheral WT, $Cbl\text{-}b^{-/-}$, $Itk^{-/-}$ and $Itk^{-/-}Cbl\text{-}b^{-/-}$ $CD4^+$ T cells were isolated, loaded with CFSE and stimulated with various concentrations of anti-CD3. Since both $Itk^{-/-}$ and $Itk^{-/-}Cbl\text{-}b^{-/-}$ mice showed a similar percentage of memory/activated $CD4^+$ T-cell phenotype, a direct comparison of total (*i.e.* naïve plus memory subsets) $CD4^+$ T cells was performed. As reported previously, $Cbl\text{-}b^{-/-}$ $CD4^+$ T cells showed hyperproliferation [12, 13], while $Itk^{-/-}$ $CD4^+$ T cells showed reduced proliferation compared with WT cells [16] (Fig. 1A). In contrast to $Itk^{-/-}$ cells, proliferation was restored in $Itk^{-/-}Cbl\text{-}b^{-/-}$ $CD4^+$ T cells (Fig. 1A). The proliferative response to anti-CD3 plus anti-CD28 stimulation was similar in all genotypes analyzed. Furthermore, the diminished IL-2 production of $Itk^{-/-}$ $CD4^+$ T cells upon costimulation [16, 32] was partially rescued in $Itk^{-/-}Cbl\text{-}b^{-/-}$ $CD4^+$ T cells (Fig. 1B). These data are in agreement with a recent report that in addition also showed that Cbl-b deletion can rescue actin polarization in $Itk^{-/-}$ T cells [27]. Itk-deficient T cells also have a Th2 polarization defect and showed impaired production of IL-4 [19, 20]. Therefore, we tested whether $Itk^{-/-}Cbl\text{-}b^{-/-}$ $CD4^+$ T cells are able to polarize toward a Th2 phenotype. Since $Itk^{-/-}Cbl\text{-}b^{-/-}$ $CD4^+$ T cells showed a tendency to produce more IL-4 compared with $Itk^{-/-}$ $CD4^+$ T cells

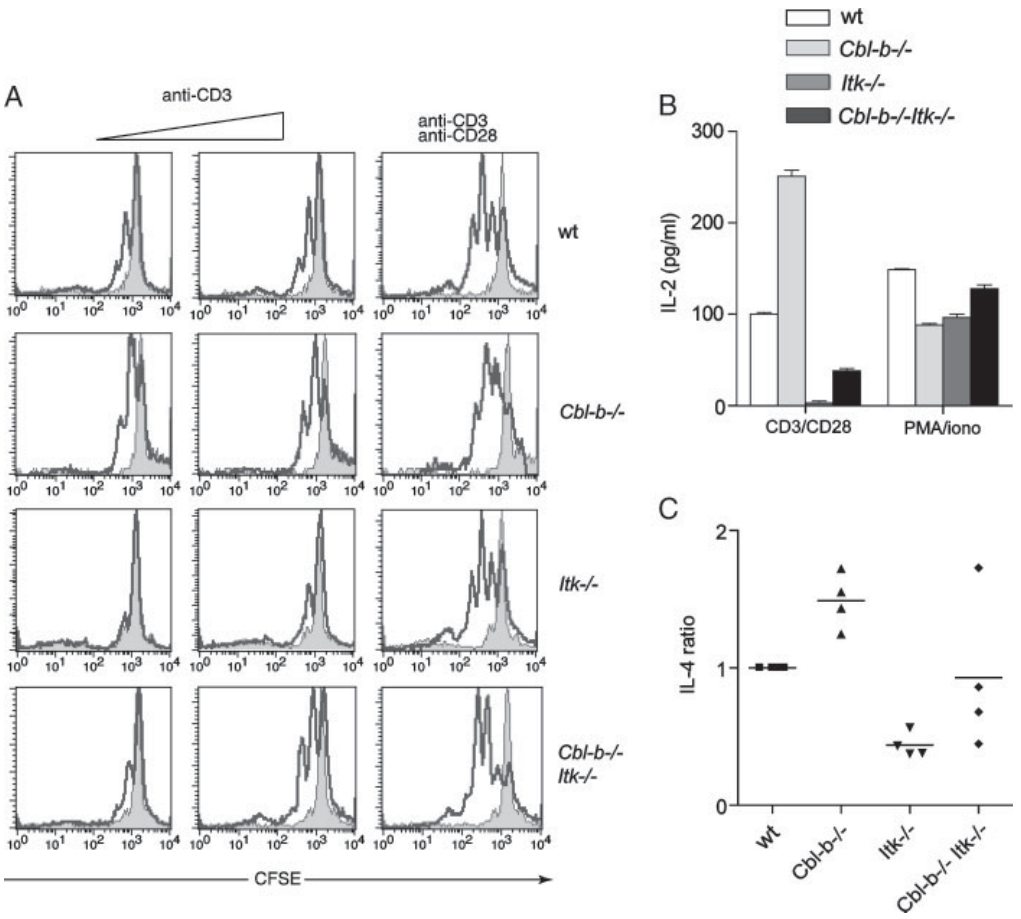

Figure 1. Deletion of Cbl-b restored defects of $Itk^{-/-}$ $CD4^+$ T cells. (A) Histograms showing fluorescence intensity of CFSE-labeled $CD4^+$ T cells of the indicated genotypes. T cells were activated with different concentrations of anti-CD3 (0.3 or 1.0 μg/mL, left or middle panel) or with anti-CD3 plus anti-CD28 (1.0 and 3.0 μg/mL, respectively, right panel). (B) $CD4^+$ T cells were activated with anti-CD3 plus anti-CD28 or with PMA and ionomycin for 48 h and IL-2 levels in the supernatant were measured by ELISA. One representative out of three independent experiments is shown. (C) $CD4^+$ T cells were polarized under Th2 conditions for 5 days. Dead cells were removed and purified Th2 cells were restimulated with anti-CD3 (3 μg/mL) for 12 h. The IL-4 concentration in the supernatant was determined by ELISA. For each experiment, IL-4 levels of WT $CD4^+$ T cells were set as one and the relative IL-4 levels of $Cbl\text{-}b^{-/-}$, $Itk^{-/-}$ and $Cbl\text{-}b^{-/-}Itk^{-/-}$ compared with WT cells were calculated. The diagram shows the results of four independent experiments.

(although with variable efficiency, Fig. 1C), the deletion of Cbl-b can also bypass the requirement of Itk for the efficient generation of IL-4-producing Th2 cells.

Lack of peripheral T cells in the absence of Itk and Vav1

The above data provide further indication that Itk and Vav1 control similar pathways in peripheral T cells, since the functional defects of both $Itk^{-/-}$ T cells and $Vav1^{-/-}$ T cells can be rescued in the absence of Cbl-b. To investigate the relation between Itk and Vav1 more directly, and to test whether the combined absence of Itk and Vav1 would reveal additional phenotypes during T-cell activation compared with single-deficient mice, $Vav1^{-/-}$ and $Itk^{-/-}$ mice were intercrossed to generate $Vav1^{-/-}Itk^{-/-}$ mice. As previously reported, both $Vav1^{-/-}$ mice [7–11] and $Itk^{-/-}$ mice [16, 17] have reduced numbers of peripheral T cells (Fig. 2A and B). In contrast, double-deficient mice displayed a dramatic loss of T cells both in lymph nodes (Fig. 2A and B) and in the spleen (data not shown). $Vav1^{-/-}Itk^{-/-}$ mice showed an approx. fourfold decrease in peripheral T-cell numbers in lymph nodes compared with Vav1 and an approx. 20-fold reduction compared with WT mice (Fig. 2B, lower panel). The number of $B220^+$ B cells was slightly increased compared with WT, $Vav1^{-/-}$ and $Itk^{-/-}$ mice (Fig. 2B).

Impaired T-cell development in $Vav1^{-/-}Itk^{-/-}$ mice

To determine whether the reduction in the peripheral T-cell pool in $Vav1^{-/-}Itk^{-/-}$ mice is caused by a defect during T-cell

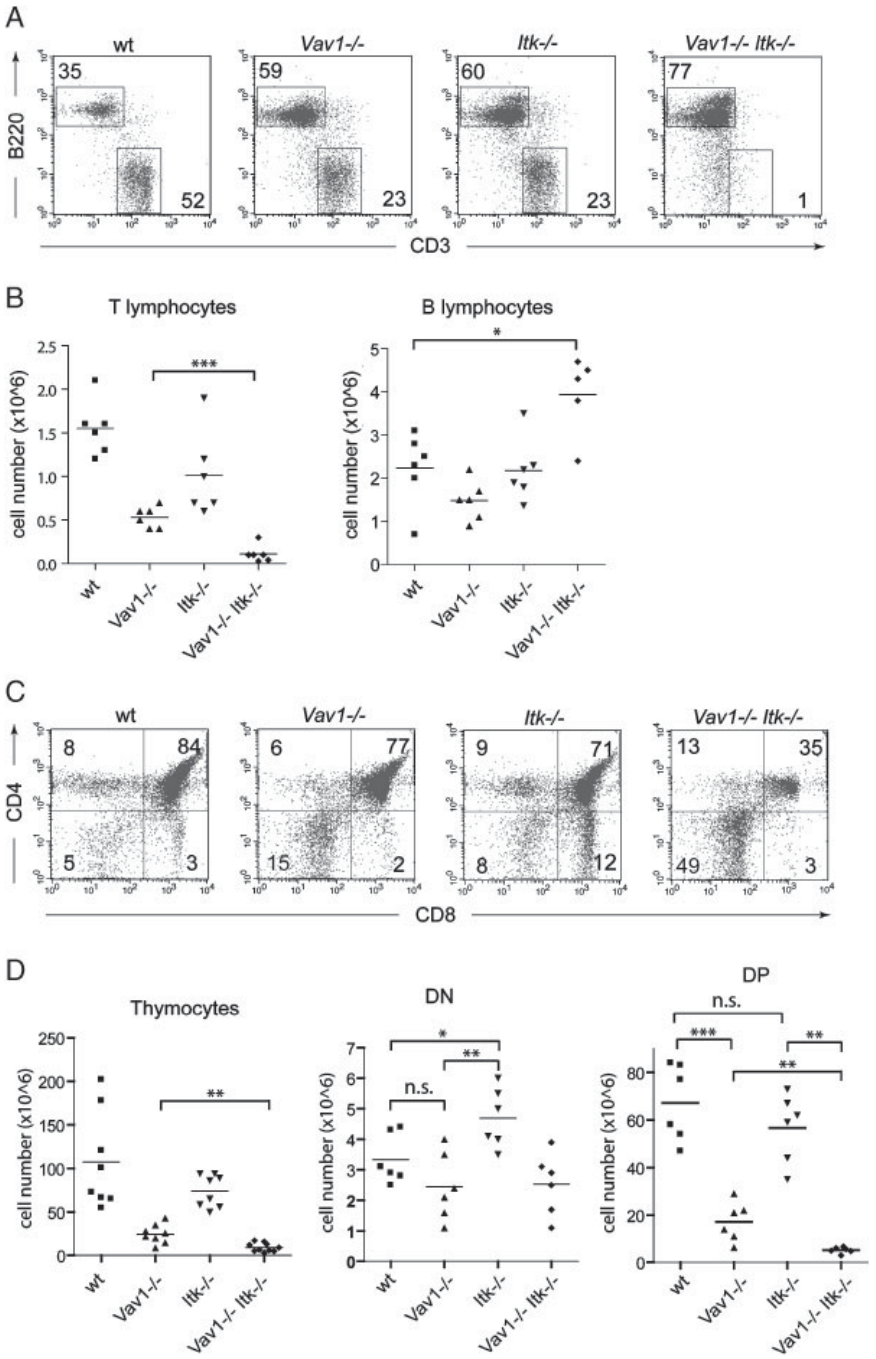

Figure 2. *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice have severely reduced numbers of peripheral T cells due to impaired thymocyte development. (A) Dot plots showing B220 and CD3 expression on lymph node cells of the indicated genotype. Numbers next to the regions indicate the percentage of cells within the gate. Each dot plot is representative of at least six mice. (B) Diagram showing the total numbers of T and B cells in lymph nodes of mice of the indicated genotype. Each data point represents one mouse. (C) Dot plots showing CD4 and CD8 expression on thymocytes of the indicated genotype. Numbers indicate the percentage of cells in the respective quadrant. Each dot plot is representative of at least six mice. (D) Diagram showing the total thymocytes numbers (left panel), total DN numbers (middle panel) or total DP numbers (right panel) in mice of the indicated genotype. Each data point represents one mouse.

development, thymocytes of the various genotypes were isolated and analyzed for the expression of CD4 and CD8. It has been shown that *Vav1*$^{-/-}$ mice have an increase in the percentage of the DN subset and reduced thymocyte cellularity [7–11], while *Itk*$^{-/-}$ mice show a defect during positive selection of both CD4 and CD8 T cells [36]. In contrast to the single knockouts, *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice displayed a severely altered CD4/CD8 expression profile, having an increase in the percentage of DN cells and a corresponding drop in the percentage of DP thymocytes (Fig. 2C). Thymic cellularity was further reduced (approx. threefold) in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice compared with *Vav1*$^{-/-}$ mice (Fig. 2D). This was due to a reduction in the numbers of DP thymocytes, since the number of DN cells was similar in WT, *Vav1*$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice. In contrast, *Itk*$^{-/-}$ mice showed a mild increase in the numbers of DN thymocytes compared with the other genotypes analyzed (Fig. 2D). However, the number of TCR-γδ T cells was similar in all genotypes (data not shown).

The developmental alteration in *Vav1*$^{-/-}$ thymocytes is caused by a partial block at the DN3 stage [10, 37]. To investigate whether the developmental phenotype seen in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice is due to a stronger but mechanistically similar defect in comparison with *Vav1*$^{-/-}$ mice, the composition of the DN subset with respect to CD44 and CD25 expression was analyzed in more detail. While WT and *Itk*$^{-/-}$ mice had a normal DN1–4 subset distribution (Fig. 3A and B), *Vav1*$^{-/-}$ showed an increase in the DN3 subset (Fig. 3A and B), as previously reported [10, 37]. *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocytes had a similar increase in the DN3 subset as *Vav1*$^{-/-}$ thymocytes, indicating that the severity of the DN3 block due to Vav1 deficiency is not further increased in the absence of Itk. To determine whether the diminished numbers of DP thymocytes is caused by a reduced proliferative capacity of DN thymocytes, BrdU labeling experiments were performed. These experiments revealed that the BrdU incorporation was similar in *Vav1*$^{-/-}$ and in *Vav1*$^{-/-}$*Itk*$^{-/-}$ DN thymocytes and DP cells and therefore indicated similar proliferation capacity (Fig. 3C). Furthermore, the experiments showed that *Vav1*$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ DN thymocytes, in particular DN3 and DN4 stages, showed even increased BrdU incorporation compared with *Itk*$^{-/-}$ DN cells, which behaved similar to their WT counterparts (Fig. 3C).

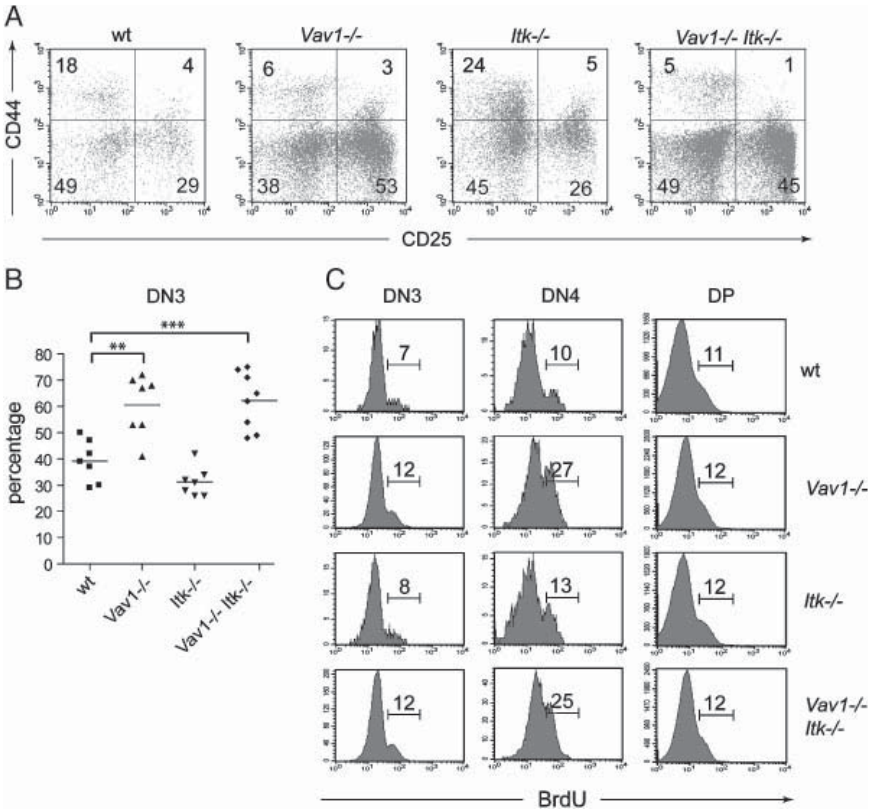

Figure 3. No developmental differences between the DN subsets of *Vav1*$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice. (A) Dot plots showing CD44 and CD25 expression on DN thymocytes of the indicated genotype. Numbers indicate the percentage of cells in the respective quadrant. Each dot plot is representative of at least six mice. (B) Diagram showing the percentage of DN3 thymocyte populations in mice of the indicated genotype. Each data point represents one mouse. (C) Histograms showing BrdU incorporation in DN3, DN4 and DP thymocytes of the indicated genotype. Mice were injected with BrdU and the incorporation of BrdU in thymocytes was analyzed 12 h later. Numbers indicate the percentage of cells within the indicated region. Each histogram is representative of at least three mice.

Reduced survival of *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocytes

Having determined that *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocytes did not show a more severe block at the DN3 stage or reduced proliferation compared with *Vav1*$^{-/-}$ cells, we tested whether the survival rate of *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocytes is altered compared with *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ thymocytes. Therefore, total thymocytes of the various genotypes were *ex vivo* analyzed with Annexin V and 7-Amino-actinomycin D (7-AAD) stainings to distinguish between early-apoptotic (Annexin V$^+$ 7-AAD$^-$) and late-apoptotic/dead (Annexin V$^+$ 7-AAD$^+$) cells. While the percentage of early-apoptotic cells was similar in WT, *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ thymocytes, it was much higher in *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocytes due to an increase in early-apoptotic cells in the DP subset (Fig. 4A and B). In contrast, the difference between *Itk*$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ cells in late-apoptotic/dead cells (Annexin V$^+$ 7-AAD$^+$) was only

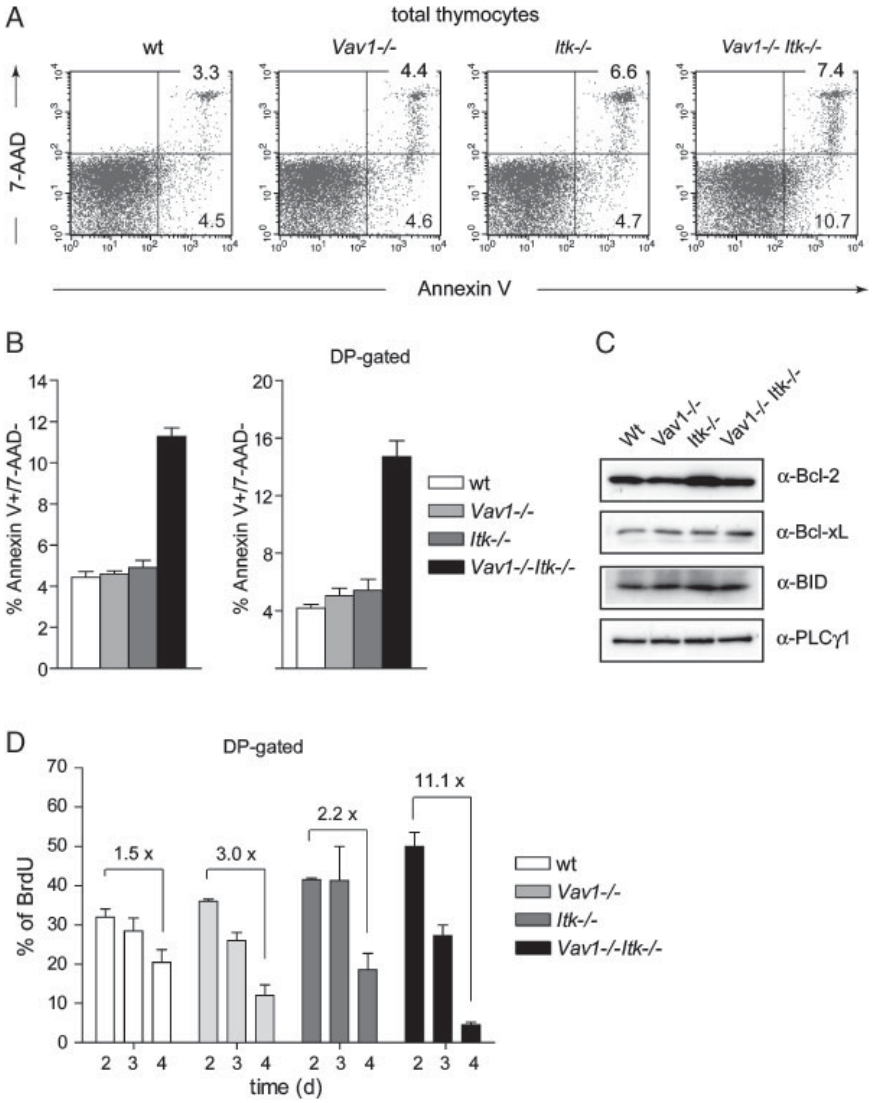

Figure 4. Increased cell death of *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP thymocytes. (A) Dot plot showing Annexin V and 7-AAD stainings of *ex vivo* isolated thymocytes of the indicated genotypes. Numbers indicate the percentage of cells in the respective quadrant. Each dot plot is representative of four mice. (B) *Ex vivo* percentages of Annexin V$^+$/7-AAD$^-$ total (left panel) or DP (right panel) thymocytes of the indicated genotypes. The diagram shows the summary of all stainings performed ($n = 4$ for each genotype). (C) Immunoblot analysis of Bcl-2, Bcl-XL and BID expression levels in purified DP thymocytes of the indicated genotype. PLC-γ1 levels were used as loading control. Data shown are representative of three different experiments. (D) Diagram showing the summary of pulse-chase BrdU labeling experiments. WT, *Vav1*$^{-/-}$, *Itk*$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice were injected with a single dose of BrdU and the percentage of BrdU$^+$ DP thymocytes was monitored on days 2, 3 and 4. For each time point, at least four WT and *Vav1*$^{-/-}$*Itk*$^{-/-}$, three *Vav1*$^{-/-}$ and two *Itk*$^{-/-}$ mice were analyzed.

moderate (Fig. 4A). These data show that *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP thymocytes have increased fractions of early-apoptotic cells. However, the expression of anti- or pro-apoptotic factors such as Bcl-2, Bcl-XL and BID was similar in WT, *Vav1*$^{-/-}$, *Itk*$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP cells (Fig. 4C).

To investigate whether the increase in Annexin V^{+} cells in the DP subset correlated with a reduced life span *in vivo*, pulse-chase BrdU labeling experiments were performed. Mice were injected with a single dose of BrdU and the percentage of BrdU^{+} cells was monitored on days 2, 3 and 4. The peak incorporation of BrdU in DP cells was observed on day 2 with approx. 30–40% of BrdU^{+} WT cells. On day 4, the number dropped to about 25%, indicating a 1.5-fold reduction compared with day 2 (Fig. 4D). Similar BrdU incorporation levels on day 2 were also observed in *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ DP cells. The numbers of BrdU^{+} *Vav1*$^{-/-}$ DP cells on day 4 dropped to about 12%, indicating a threefold reduction compared with day 2 and a moderate reduction in the half-life compared with WT DP cells (Fig. 4D). *Itk*$^{-/-}$ DP cells had approx. 18% of BrdU^{+} *Vav1*$^{-/-}$ DP cells on day 4 (a 2.1-fold reduction). Within the *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP thymocyte subsets, about 50% of the cells were BrdU^{+} on day 2 (Fig. 4D). The higher percentage of BrdU^{+} cells compared with WT DP thymocytes is most likely a consequence of the increased BrdU incorporation observed in DN3/DN4 cells on day 1 (Fig. 3C). However, on day 4 the number of BrdU^{+} *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP cells dropped to 4–5%, leading to an 11-fold reduction in BrdU^{+} cells (Fig. 4D). This indicates that the *in vivo* life span of *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP cells was dramatically reduced compared with WT as well as *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ DP thymocytes.

Impaired positive selection of *Vav1*$^{-/-}$ *Itk*$^{-/-}$ thymocytes

Despite the reduced percentage of DP thymocytes, CD4 and CD8 SP thymocytes were still present in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice, although in reduced numbers (Fig. 2C and D). To characterize the SP and also the DP subsets in more detail, the expression levels of CD3 and CD5 were determined. The majority of WT and *Itk*$^{-/-}$ CD4 SP thymocytes had high levels of CD3 and CD5 and therefore were of a mature phenotype; only approx. 50% of *Vav1*$^{-/-}$ CD4 SP cells had high levels of CD3 and CD5 (Fig. 5A and B). This indicates an aberrant population of CD4 SP cells in *Vav1*$^{-/-}$ mice. In contrast, there were essentially no CD3^{hi} or CD5^{hi} cells in the CD4 SP subset in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice (Fig. 5A and B). A lack of CD3^{hi} but not of CD5^{hi} cells was also observed within the CD8 SP population (Fig. 5A and B).

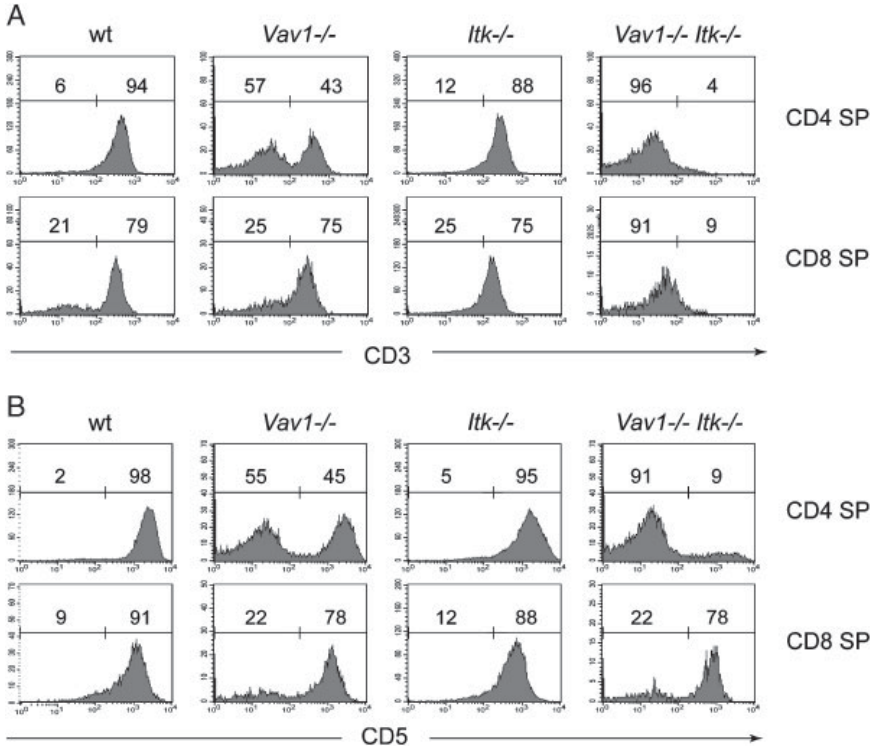

Figure 5. *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice lack mature SP thymocytes. Histograms showing CD3 (A) and CD5 (B) expression on CD4 SP and CD8 SP thymocytes of the indicated genotype. Numbers indicate the percentage of cells within the indicated region. Each histogram is representative of at least six mice.

To evaluate whether the loss of mature SP cells in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice is caused by impaired positive selection, the CD69 expression pattern on DP and SP thymocytes was determined. CD69 is up-regulated upon TCR triggering on DP thymocytes at the onset of positive selection and is subsequently down-regulated through the progression to the SP stage [38–40]. While there was a reduction in CD69$^+$ DP cells in *Vav1*$^{-/-}$ mice compared with WT controls [10], there was no further reduction in CD69$^+$ DP cells in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice compared with *Vav1*$^{-/-}$ mice (data not shown). This is in contrast to SP cells, since the CD69$^+$ population in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice was almost completely absent, while TCRβ^{hi} CD4 and CD8 SP cells that still expressed CD69 could be readily detected in WT, *Itk*$^{-/-}$ and also in *Vav1*$^{-/-}$ SP thymocytes (Fig. 6A and B). To further analyze whether positive selection is impaired in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice, the Ca^{2+} mobilization in DP thymocytes upon triggering of the TCR was determined. While the Ca^{2+} response was reduced in *Itk*$^{-/-}$ and *Vav1*$^{-/-}$ DP thymocytes compared with WT cells (WT > *Itk*$^{-/-}$ > *Vav1*$^{-/-}$), it was almost completely abolished in *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP thymocytes (Fig. 6C). Together, these data indicate a block in positive selection of DP thymocytes in the combined absence of Vav1 and Itk.

Itk$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ CD8 SP thymocytes have a memory phenotype

Finally, it has been shown that Itk and Rlk are essential for the development of conventional CD8$^+$ T cells. As a consequence, *Itk*$^{-/-}$ mice and *Itk*$^{-/-}$*Rlk*$^{-/-}$ mice are almost devoid of conventional CD8$^+$ T cells, while the remaining CD8$^+$ T-cell subsets have a memory marker expression phenotype and several characteristics of innate-like T lymphocytes, such as the up-regulation of CD44 and CD122, the ability to produce rapidly IFN-γ upon stimulation and their dependency on IL-15 for their survival [33–35, 41]. Therefore, the CD44 and CD122 as well as heat-stable antigen (HSA) expression phenotype of *Vav1*$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ CD8 SP thymocytes was determined. The majority of WT and *Vav1*$^{-/-}$ CD8 SP cells already contained a large fraction of HSA^{lo} cells and were CD44^{lo} and CD122^{lo} (Fig. 7), while a large population of *Itk*$^{-/-}$ CD8 SP cells as previously shown [33–35, 41] were CD44^{hi}, CD122^{hi} and HSA^{lo} (Fig. 7). The few remaining CD8 SP cells in *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocytes showed even a larger fraction of CD44^{hi}CD122^{hi} cells compared with *Itk*$^{-/-}$ thymocytes; however, the HSA^{lo}/HSA^{hi} distribution was similar to that of WT and *Vav1*$^{-/-}$ CD8 SP cells.

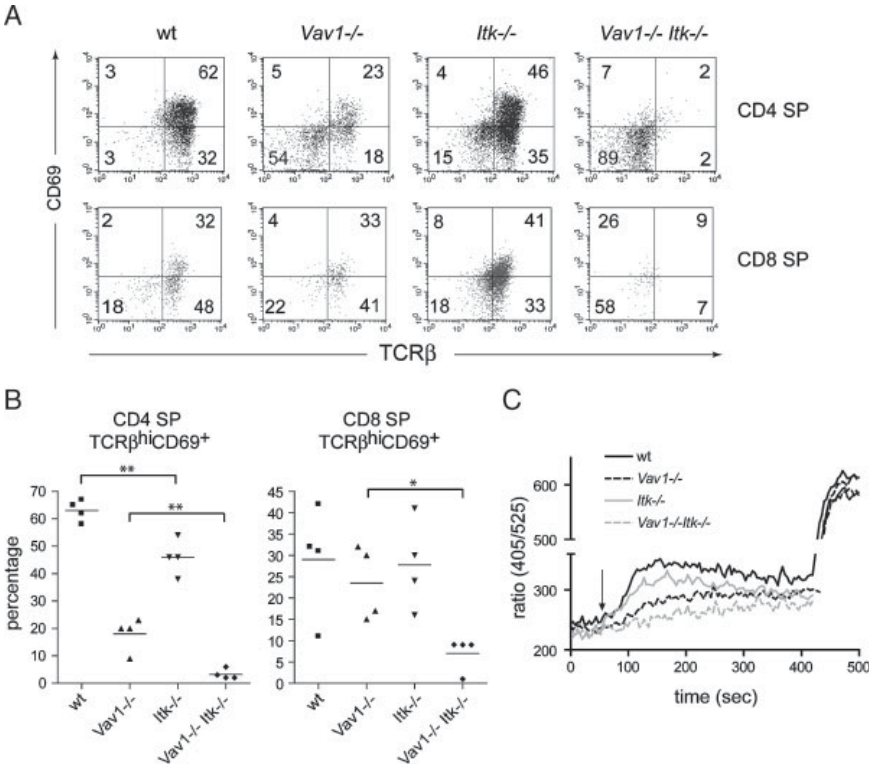

Figure 6. Impaired positive selection of *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP thymocytes. (A) Dot plots showing the percentage of TCRβ^{hi}CD69$^+$ CD4 and CD8 SP thymocytes of the indicated genotype. Numbers indicate the percentage of cells in the respective quadrant. (B) Diagram showing the summary of panel (A). Each data point represents one mouse ($n = 4$). (C) Time course showing Ca^{2+} response (measured as fluorescence 405/525 nm ratio) of DP thymocytes of the indicated genotype that were labeled with Indo-1. Thymocytes were incubated for 1 min with biotinylated anti-CD3 (1 μg/mL) and fully activated by crosslinking with streptavidin (indicated by the arrow). One representative experiment out of four is shown.

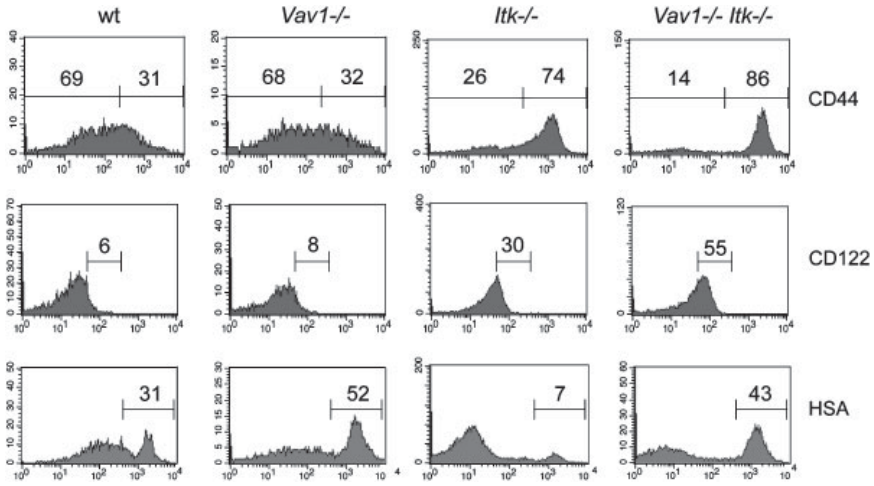

Figure 7. Itk-deficient but not Vav1-deficient CD8 SP thymocytes have a memory phenotype. CD44 (upper panel), CD122 (middle panel) and HSA (lower panel) expressions on WT, *Vav1*$^{-/-}$, *Itk*$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ CD8 SP thymocytes. Numbers in the histogram indicate the percentage of cells within the region. Data are representative of two mice, except for *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocytes ($n = 4$).

Thus, the CD44 and CD122 expression profile of CD8 SP thymocytes indicated that Vav1, unlike Itk, is not essential for the development of conventional CD8$^+$ T cells.

Discussion

Existing crosstalks between Vav1 and Itk and the similar phenotype of *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ T cells suggest that they act in the same signaling pathways and perform (at least partially) similar functions in activated T cells. In this study we further investigated potential interdependences and similarities between Itk and Vav1. To investigate whether the defects in peripheral *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ T cells are mechanistically similar, we tested whether the defect of *Itk*$^{-/-}$ T cells is rescued upon deletion of the E3 ubiquitin ligase Cbl-b, as described for *Vav1*$^{-/-}$ T cells [15]. The analysis of *Itk*$^{-/-}$*Cbl-b*$^{-/-}$ mice revealed partially restored T-cell function if *Itk*$^{-/-}$ T cells are released from the negative regulation mediated by Cbl-b. Thus, our data provide additional evidence that Vav1 and Itk fulfill similar functions in peripheral T cells. In addition, we wanted to address genetically whether there is functional redundancy between Vav1 and Itk during T-cell activation by generating Vav1/Itk double-deficient mice. However, *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice showed severely reduced numbers of peripheral T cells due to increased cell death of DP thymocytes and impaired positive selection, thus indicating an essential role for the combined activity of Itk and Vav1 during T-cell development.

The defect in Vav1-deficient T cells is rescued upon deletion of the E3 ubiquitin ligase Cbl-b, which mechanistically bypasses the requirement for a CD28 costimulatory signal for the efficient formation of the immunological synapse [15]. Therefore, we tested whether the phenotype of *Itk*$^{-/-}$ T cells is rescued if Cbl-b is deleted. An interaction between Vav1 and Itk has been demonstrated [21] and Itk has been shown to regulate the actin cytoskeleton [4, 31]. Thus, it was not surprising that we observed, in agreement with another study [27], a rescue of the proliferation and IL-2 production defect of *Itk*$^{-/-}$ T cells in the absence of Cbl-b. Furthermore, we also observed a partial rescue of IL-4 production in Th2 polarized *Itk*$^{-/-}$*Cbl-b*$^{-/-}$ CD4$^+$ T cells compared with IL-4 of *Itk*$^{-/-}$ CD4$^+$ T cells. It has also been shown that the Ca^{2+} response is still impaired in *Itk*$^{-/-}$*Cbl-b*$^{-/-}$ T cells, as observed for *Vav1*$^{-/-}$*Itk*$^{-/-}$ cells [27]. This indicates that loss of Cbl-b effects pathways independent or downstream of Ca^{2+} mobilization. It is likely that the rescue of Itk-deficient T cells upon loss of Cbl-b is due to effects on the actin cytoskeleton, since it has been demonstrated that loss of Cbl-b rescues actin polymerization in *Itk*$^{-/-}$ T cells in response to anti-CD3-coated beads [27]. In line with this observation, lipid raft recruitment (which is dependent on actin) to the site of anti-TCR or anti-TCR/CD28 beads was improved in *Itk*$^{-/-}$*Cbl-b*$^{-/-}$ T cells compared with Itk-deficient cells [27]. A similar observation has been made in *Vav1*$^{-/-}$*Cbl-b*$^{-/-}$ T cells [15]. Thus, the rescue appears mechanistically similar to *Vav1*$^{-/-}$*Cbl-b*$^{-/-}$ T cells. Together, these data strengthen the evidence that Itk and Vav1 have (partial) similar functions in peripheral T cells and reveal additional mechanistic similarities between *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ T cells.

The CD4 and CD8 expression profile of developing thymocytes in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice is reminiscent of the one described for mice deficient for all three members (Vav1, Vav2 and Vav3) of the Vav family. Vav1,2,3-null mice show a severe block at the DN3 stage during T-cell development and it was reported that there is a 50–100-fold reduction in thymocyte numbers [37]. However, it has not been reported whether Vav1,2,3-null DP thymocytes show impaired survival compared with WT cells. Among the Vav family, Vav1 has been shown to be the most important member with respect to T-cell development, since Vav1-deficient mice showed already impaired thymocyte development due to a block at the DN3 to DN4 stage [10, 11].

In contrast to *Vav1*$^{-/-}$ mice, *Itk*$^{-/-}$ mice do not show any developmental defect within the DN subsets [36]. A recent study comparing WT and *Itk*$^{-/-}$ thymocytes in mixed BM chimeras revealed a slightly impaired ability of *Itk*$^{-/-}$ thymocytes to reconstitute the DP subset due to subtle defects in pre-TCR signaling [42], although we did not observe differences in the incorporation of BrdU between WT and *Itk*$^{-/-}$ DN3, DN4 or DP thymocytes. The discrepancy between these results is most likely caused due to the different experimental settings in which either a competitive or a non-competitive development of *Itk*$^{-/-}$ thymocytes in mixed BM chimeras or knockout mice, respectively, was analyzed. The DN cell numbers, the DN1–4 profile and the proliferation rate of DN3, DN4 and DP thymocytes (as measured by BrdU incorporation) were similar in *Vav1*$^{-/-}$ and *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice. Thus, the dramatic loss of DP cells in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice cannot be simply explained by a stronger phenotype due to a more severe block at the DN3 stage in *Vav1*$^{-/-}$*Itk*$^{-/-}$ thymocytes compared with *Vav1*$^{-/-}$ thymocytes. We rather favor the explanation that the combined deletion of Itk and Vav1 alters intracellular signaling pathways that are required for the survival of DP thymocytes, since *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP cells had a reduced life span *in vivo* compared with WT as well as *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ DP cells. Whether the survival signals are transmitted by pre-TCR signals or *via* cytokine receptor signaling or both remains to be determined. Although we clearly observed an increase in early-apoptotic cells in *Vav1*$^{-/-}$*Itk*$^{-/-}$ compared with *Vav1*$^{-/-}$ DP thymocytes, the expression of pro- or anti-apoptotic signaling molecules such as BclXL, Bcl-2 and BID was indistinguishable between all genotypes analyzed. The severely reduced numbers of peripheral T cells are most likely a consequence of the diminished numbers of DP cells. However, the remaining *Vav1*$^{-/-}$*Itk*$^{-/-}$ DP thymocytes showed also a defect in positive selection, suggested by a further reduction in the Ca^{2+} response in DP cells and the absence of CD69^{+} SP thymocytes compared with *Vav1*$^{-/-}$ cells. Preliminary results also indicated that Erk1/2 phosphorylation in *Vav1*$^{-/-}$*Itk*$^{-/-}$ was as severely reduced as observed in *Vav1*$^{-/-}$ DP cells (data not shown). Thus, the combined activity of Vav1 and Itk is essential for the survival of DP thymocytes and for positive selection.

A connecting molecule between Vav1 and Itk may be the adaptor protein SLP76, which has been shown to interact with both Vav1 and Itk [43]. The Vav1 interaction depends on SLP76 N-terminal tyrosine residues Y112 and Y128, while for the interaction with Itk Y145 is required [44]. SPL76 is essential for the transition from DN to DP thymocytes, since *SLP76*$^{-/-}$ mice have a severe block at the DN3 stage [45, 46]. While the thymocyte phenotype of *SLP76*$^{-/-}$ mice is completely rescued by transgenic expression of WT SLP76, transgenic expression of a triple Y112-128-145F (Y3F) mutant SLP76 only partially rescued the SLP76-deficient phenotype. Y3F mice on an SLP76 "knockout" background (Y3F/ΔSLP76) developed DP but only few mature SP thymocytes showed impaired up-regulation of CD69 and the thymic cellularity was reduced by 90% [47, 48]. Thus, the Y3F/ΔSLP76 phenotype partially resembles the thymocyte defect observed in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice. However, the *Vav1*$^{-/-}$*Itk*$^{-/-}$ phenotype is more severe, since the percentage of DP cells was much lower and peripheral T cells were severely diminished compared with Y3F/ΔSLP76 mice. Further evidence that SLP76 may serve as a link between Vav1 and Itk was obtained from "knock-in" mutations of the respective tyrosine residues (Y to F), since the activation of Vav1 or Itk was differentially affected by the mutations. The phenotype of Y112-128F T cells was very similar to the *Vav1*$^{-/-}$ phenotype, while Y145F mice resembled the Itk-deficient phenotype, including the appearance of increased percentages of innate-like CD8^{+} T cells [49]. Although the lack of Y122/128 or Y145 impaired the activation of Vav1 or Itk, respectively, Vav1 or Itk were still able to interact with SLP76 [49]. Therefore, both molecules were still part of the TCR signalosome and could partially function even in the absence of activation. This may also provide a potential explanation as to why the thymocyte phenotype of Y3F/ΔSLP76 mice appears to be weaker compared with *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice.

Vav proteins are the GDP/GTP exchange factor that activates members of the Rho GTPases family, while Itk is a protein tyrosine kinase. However, the similarities in the T-cell development phenotype of Vav1,2,3-null mice and Vav1/Itk double-deficient mice may suggest similar functions despite different enzymatic activities, as indicated already for peripheral T cells (see [27] for discussion). It has even been shown that Itk is required in a kinase-independent way for the recruitment of Vav1 to the LAT/SLP76 complex [21]. To test whether Itk has a kinase-independent activity (*i.e.* a potential scaffolding function) also during T-cell development, we initiated BM reconstitution experiments expressing Itk or a kinase-dead version of Itk (Itk-KD). Unfortunately, transduced *Vav1*$^{-/-}$*Itk*$^{-/-}$ BM cells reconstituted the hematopoietic system of lethally irradiated WT mice with very low efficiency (J. R. and W. E., unpublished observation). From more than 40 reconstituted mice we were able to generate only one Itk-KD and one Itk-BM chimeric mouse. Both Itk-KD and Itk could rescue the appearance of DP cells, while the kinase activity of Itk was required to rescue the appearance of mature SP thymocytes (see Supporting Information). This preliminary result suggests different functional activities of Itk during T-cell development (in the absence of Vav1), *i.e.* a scaffolding function of Itk for the generation of DP cells, while the kinase activity of Itk is essential for positive selection of DP thymocytes.

Despite the crosstalks between Vav1 and Itk, and the phenotypic similarities of Vav1- and Itk-deficient peripheral T cells, there is a different requirement for Vav1 and Itk during T-cell development. As mentioned above, *Vav1*$^{-/-}$ DN thymocytes show a partial block at the DN3 stage, while *Itk*$^{-/-}$ DN cells appear to be relatively normal. As a consequence, *Vav1*$^{-/-}$ mice have reduced percentages of DP cells compared with *Itk*$^{-/-}$ mice. Furthermore, it has been recently shown that Itk is essential for conventional T-cell differentiation [50]. Therefore, the majority of CD8^{+} T cells as well as an increased percentage of CD4^{+} T cells in *Itk*$^{-/-}$ mice have a CD44^{hi}CD122^{hi} memory phenotype and characteristics of innate-like T cells [33–35, 41, 51]. These innate-like T cells develop in the thymus and therefore CD8 SP

display already a $CD44^{hi}CD122^{hi}$ phenotype [33, 34]. Although we also observed an increase in $CD44^{hi}$ cells in *Vav1*$^{-/-}$ peripheral $CD8^+$ T cells (data not shown), CD8 SP thymocytes in Vav1-deficient mice were similar to WT CD8 SP cells. Together, these data clearly indicate that several processes during T-cell development are uniquely regulated by either Vav1 or Itk.

In summary, our study reveals additional mechanistic similarities between the T-cell activation phenotype of *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ T cells and indicates an essential role for the combined activity of Vav1 and Itk for the survival of DP thymocytes and positive selection during T-cell development.

Materials and methods

Mice

Animals used in this study were 6–12 wk of age and bred and maintained in the animal facility of the Medical University of Vienna. *Vav1*$^{-/-}$ [11], *Itk*$^{-/-}$ [16] and *Cbl-b*$^{-/-}$ [12] mice were previously described and backcrossed to C57BL/6 (>N10). Animal experiments were approved by the Federal Ministry for Science and Research.

Flow cytometric analysis

All flow cytometric analyses were performed on a FACSCalibur (BD Biosciences). The following antibodies were from BD Pharmingen: FITC-anti-CD3 (145-2C11), FITC-anti-CD44 (IM7), PE-anti-CD5 (53-7.3), PE-anti-TCR-γδ (GL3), PE-anti-CD122, FITC-anti-HSA (M1/69); the following antibodies were from Caltag: TC-anti-CD4 (CT-CD4), A647-anti-CD3, FITC-anti-CD45R (RA3-6B2), FITC-anti-CD69 (H1.2FF3), PE-anti-CD25 (PC61 5.3); APC-anti-CD8α (53-6.7) and PE-anti-CD44 (IM7) were used from eBioscience.

BrdU incorporation and pulse-chase experiments

Mice were injected intraperitoneally with 1 mg (in 200 μL) BrdU. After various time points, thymocytes were isolated, stained for surface markers, and analyzed using the BrdU flow kit (BD Pharmingen).

Annexin V and 7-AAD analysis

Thymocytes were stained for CD4 and CD8 and later incubated in Annexin-binding buffer (1×10^6 cells/100 μL) containing 5 μL of FITC-Annexin V (Caltag) and 5 μL of 7-AAD (BD) for 20 min at room temperature. The percentage of apoptotic or dead cells was determined by flow cytometry.

Immunoblotting

DP thymocytes (5×10^6) were lyzed in 100 μL of Carin lysis buffer (20 mM Tris-HCl, pH 8.0, 138 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP40, 10% Glycerol and 2 mM NA-Vanadate) supplemented with complete protease inhibitors (Roche). Proteins were then separated on 8% SDS-polyacrylamide gels and blotted onto PVDF membranes (Biorad). Anti- Bcl-2 (C-2), anti-Bcl-XL (H-62) and anti-BID (D-19) were purchased from Santa Cruz. Anti-PLCg1 was from Cell Signalling. Secondary antibodies were from Jackson Immunoresearch.

Purification of DP thymocytes

Thymocytes were stained with CD8α APC (53-6.7, eBioscience) and purified *via* positive selection using anti-APC Beads (MACS System, Miltenyi Biotec) according to the manufacture's instructions. The purity of cells was assessed by flow cytometry and was routinely >90%.

Purification of $CD4^+$ T cells

Pooled cell suspensions of lymph nodes and spleens were incubated with biotinylated anti-CD8α, anti-CD11b, anti-CD11c, anti-CD45R, anti-Ly-6G, anti-Ter119 and anti-NK antibodies in PBS supplemented with 2% FBS. The $CD4^+$ T cells were then purified by negative depletion using streptavidin beads (BD Pharmingen) according to the manufacture's instructions. The purity of the cells was assessed by flow cytometry and was routinely >96%.

Calcium mobilization

Thymocytes were stained for CD4 and CD8 and labeled (1×10^6/mL) for 30 min with Indo-1 (1 μM, Molecular Probes) and the Ca^{2+}-dependent fluorescence of Indo-1 was assessed by flow cytometry. Cells were stimulated with soluble bio-anti-CD3ε (1 μg/mL) followed by crosslinking with 5 μg/mL of streptavidin (Sigma). Overall Ca^{2+} content was assessed by the addition of ionomycin (Sigma) at the end of each measurement. Data were analyzed using CELLQuest (Becton Dickinson) and FlowJo (TreeStar) software.

T-cell proliferation assay

Purified $CD4^+$ T cells were labeled with CFSE (Molecular Probes) and cultured at 5×10^4 cells/well in 96-well flat-bottom plates (NUNC) coated with anti-CD3ε (0, 0.3 or 1 μg/mL) in a total of 100 μL. Additional wells were coated with anti-CD3ε (1 μg/mL) plus anti-CD28 (3 μg/mL); 48 h later, the cells were harvested and analyzed by flow cytometry.

Differentiation of T helpers

Purified $CD4^+$ T cells were stimulated with plate-bound anti-CD3ε (1 μg/mL) and plate-bound anti-CD28 (3 μg/mL) on 48-well plates. For Th2 polarizing conditions, cells were activated in the presence of recombinant IL-4 (250 U/mL), anti-IFN-γ (10 μg/mL) and anti-IL12 (10 μg/mL). $CD4^+$ T-cell cultures were split 1:2 on day 3 after activation. After 6 days in culture, cells were purified over a lymphoprep gradient and restimulated (5×10^5 cells/mL) with plate-bound anti-CD3ε (0.1 μg/mL). Supernatant for cytokine quantification was collected 12 h later.

Cytokine measurement

IL-2 and IL-4 cytokine levels were determined by ELISA (BD).

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed by using Student's *t*-test ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; n.s., not significant).

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References

1 **Samelson, L. E.,** Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. *Annu. Rev. Immunol.* 2002. **20**: 371–394.

2 **Davis, D. M. and Dustin, M. L.,** What is the importance of the immunological synapse? *Trends Immunol.* 2004. **25**: 323–327.

3 **Krawczyk, C. and Penninger, J. M.,** Molecular controls of antigen receptor clustering and autoimmunity. *Trends Cell Biol.* 2001. **11**: 212–220.

4 **Labno, C. M., Lewis, C. M., You, D., Leung, D. W., Takesono, A., Kamberos, N., Seth, A. and Finkelstein, L. D.,** Itk functions to control actin polymerization at the immune synapse through localized activation of Cdc42 and WASP. *Curr. Biol.* 2003. **13**: 1619–1624.

5 **Cantrell, D.,** Commentary: Vav-1 and T cells. *Eur. J. Immunol.* 2003. **33**: 1070–1072.

6 **Tybulewicz, V. L.,** Vav-family proteins in T-cell signalling. *Curr. Opin. Immunol.* 2005. **17**: 267–274.

7 **Fischer, K. D., Zmuldzinas, A., Gardner, S., Barbacid, M., Bernstein, A. and Guidos, C.,** Defective T-cell receptor signalling and positive selection of Vav-deficient CD4+ CD8+ thymocytes. *Nature* 1995. **374**: 474–477.

8 **Tarakhovsky, A., Turner, M., Schaal, S., Mee, P. J., Duddy, L. P., Rajewsky, K. and Tybulewicz, V. L.,** Defective antigen receptor-mediated proliferation of B and T cells in the bsence of Vav. *Nature* 1995. **374**: 467–470.

9 **Zhang, R., Alt, F. W., Davidson, L., Orkin, S. H. and Swat, W.,** Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. *Nature* 1995. **374**: 470–473.

10 **Turner, M., Mee, P. J., Walters, A. E., Quinn, M. E., Mellor, A. L., Zamoyska, R. and Tybulewicz, V. L.,** A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. *Immunity* 1997. **7**: 451–460.

11 **Fischer, K. D., Kong, Y. Y., Nishina, H., Tedford, K., Marengere, L. E., Kozieradzki, I., Sasaki, T. and Starr, M.,** Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor. *Curr. Biol.* 1998. **8**: 554–562.

12 **Bachmaier, K., Krawczyk, C., Kozieradzki, I., Kong, Y. Y., Sasaki, T., Oliveira-dos-Santos, A., Mariathasan, S. and Bouchard, D.,** Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* 2000. **403**: 211–216.

13 **Chiang, Y. J., Kole, H. K., Brown, K., Naramura, M., Fukuhara, S., Hu, R. J., Jang, I. K. and Gutkind, J. S.,** Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* 2000. **403**: 216–220.

14 **Loeser, S. and Penninger, J. M.,** Regulation of peripheral T cell tolerance by the E3 ubiquitin ligase Cbl-b. *Semin. Immunol.* 2007. **19**: 206–214.

15 **Krawczyk, C., Bachmaier, K., Sasaki, T., Jones, R. G., Snapper, S. B., Bouchard, D., Kozieradzki, I. and Ohashi, P. S.,** Cbl-b is a negative regulator of receptor clustering and raft aggregation in T cells. *Immunity* 2000. **13**: 463–473.

16 **Liao, X. C. and Littman, D. R.,** Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. *Immunity* 1995. **3**: 757–769.

17 **Liu, K. Q., Bunnell, S. C., Gurniak, C. B. and Berg, L. J.,** T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. *J. Exp. Med.* 1998. **187**: 1721–1727.

18 **Schwartzberg, P. L., Finkelstein, L. D. and Readinger, J. A.,** TEC-family kinases: regulators of T-helper-cell differentiation. *Nat. Rev. Immunol.* 2005. **5**: 284–295.

19 **Fowell, D. J., Shinkai, K., Liao, X. C., Beebe, A. M., Coffman, R. L., Littman, D. R. and Locksley, R. M.,** Impaired NFATc translocation and failure of Th2 development in Itk-deficient CD4+ T cells. *Immunity* 1999. **11**: 399–409.

20 **Schaeffer, E. M., Yap, G. S., Lewis, C. M., Czar, M. J., McVicar, D. W., Cheever, A. W., Sher, A. and Schwartzberg, P. L.,** Mutation of Tec family kinases alters T helper cell differentiation. *Nat. Immunol.* 2001. **2**: 1183–1188.

21 **Dombroski, D., Houghtling, R. A., Labno, C. M., Precht, P., Takesono, A., Caplen, N. J., Billadeau, D. D. and Wange, R. L.,** Kinase-independent functions for Itk in TCR-induced regulation of Vav and the actin cytoskeleton. *J. Immunol.* 2005. **174**: 1385–1392.

22 **Crespo, P., Schuebel, K. E., Ostrom, A. A., Gutkind, J. S. and Bustelo, X. R.,** Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature* 1997. **385**: 169–172.

23 **Heyeck, S. D., Wilcox, H. M., Bunnell, S. C. and Berg, L. J.,** Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity. *J. Biol. Chem.* 1997. **272**: 25401–25408.

24 **Bunnell, S. C., Henry, P. A., Kolluri, R., Kirchhausen, T., Rickles, R. J. and Berg, L. J.,** Identification of Itk/Tsk Src homology 3 domain ligands. *J. Biol. Chem.* 1996. **271**: 25646–25656.

25 **Bustelo, X. R., Crespo, P., Lopez-Barahona, M., Gutkind, J. S. and Barbacid, M.,** Cbl-b, a member of the Sli-1/c-Cbl protein family, inhibits Vav-mediated c-Jun N-terminal kinase activation. *Oncogene* 1997. **15**: 2511–2520.

26 **Marengere, L. E., Mirtsos, C., Kozieradzki, I., Veillette, A., Mak, T. W. and Penninger, J. M.,** Proto-oncoprotein Vav interacts with c-Cbl in activated thymocytes and peripheral T cells. *J. Immunol.* 1997. **159**: 70–76.

27 **Gomez-Rodriguez, J., Readinger, J. A., Viorritto, I. C., Mueller, K. L., Houghtling, R. A. and Schwartzberg, P. L.,** Tec kinases, actin, and cell adhesion. *Immunol. Rev.* 2007. **218**: 45–64.

28 **Berg, L. J., Finkelstein, L. D., Lucas, J. A. and Schwartzberg, P. L.,** Tec family kinases in T lymphocyte development and function. *Annu. Rev. Immunol.* 2005. **23**: 549–600.

29 **Reynolds, L. F., Smyth, L. A., Norton, T., Freshney, N., Downward, J., Kioussis, D. and Tybulewicz, V. L.,** Vav1 transduces T cell receptor signals to the activation of phospholipase C-gamma1 via phosphoinositide 3-kinase-dependent and -independent pathways. *J. Exp. Med.* 2002. **195**: 1103–1114.

30 **Finkelstein, L. D., Shimizu, Y. and Schwartzberg, P. L.,** Tec kinases regulate TCR-mediated recruitment of signaling molecules and integrin-dependent cell adhesion. *J. Immunol.* 2005. **175**: 5923–5930.

31 **Grasis, J. A., Browne, C. D. and Tsoukas, C. D.,** Inducible T cell tyrosine kinase regulates actin-dependent cytoskeletal events induced by the T cell antigen receptor. *J. Immunol.* 2003. **170**: 3971–3976.

32 **Li, C. R. and Berg, L. J.,** Itk is not essential for CD28 signaling in naive T cells. *J. Immunol.* 2005. **174**: 4475–4479.

33 **Atherly, L. O., Lucas, J. A., Felices, M., Yin, C. C., Reiner, S. L. and Berg, L. J.,** The Tec family tyrosine kinases Itk and Rlk regulate the development of conventional CD8+ T cells. *Immunity* 2006. **25**: 79–91.

34 **Broussard, C., Fleischacker, C., Horai, R., Chetana, M., Venegas, A. M., Sharp, L. L., Hedrick, S. M. and Fowlkes, B. J.,** Altered development of CD8+ T cell lineages in mice deficient for the Tec kinases Itk and Rlk. *Immunity* 2006. **25**: 93–104.

35 **Hu, J., Sahu, N., Walsh, E. and August, A.,** Memory phenotype CD8+ T cells with innate function selectively develop in the absence of active Itk. *Eur. J. Immunol.* 2007. **37**: 2892–2899.

36 **Schaeffer, E. M., Broussard, C., Debnath, J., Anderson, S., McVicar, D. W. and Schwartzberg, P. L.,** Tec family kinases modulate thresholds for thymocyte development and selection. *J. Exp. Med.* 2000. **192**: 987–1000.

37 **Fujikawa, K., Miletic, A. V., Alt, F. W., Faccio, R., Brown, T., Hoog, J., Fredericks, J. and Nishi, S.,** Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. *J. Exp. Med.* 2003. **198**: 1595–1608.

38 **Bendelac, A., Matzinger, P., Seder, R. A., Paul, W. E. and Schwartz, R. H.,** Activation events during thymic selection. *J. Exp. Med.* 1992. **175**: 731–742.

39 **Swat, W., Dessing, M., von Boehmer, H. and Kisielow, P.,** CD69 expression during selection and maturation of CD4+8+ thymocytes. *Eur. J. Immunol.* 1993. **23**: 739–746.

40 **Merkenschlager, M., Graf, D., Lovatt, M., Bommhardt, U., Zamoyska, R. and Fisher, A. G.,** How many thymocytes audition for selection? *J. Exp. Med.* 1997. **186**: 1149–1158.

41 **Dubois, S., Waldmann, T. A. and Muller, J. R.,** ITK and IL-15 support two distinct subsets of CD8+ T cells. *Proc. Natl. Acad. Sci. USA* 2006. **103**: 12075–12080.

42 **Lucas, J. A., Felices, M., Evans, J. W. and Berg, L. J.,** Subtle defects in pre-TCR signaling in the absence of the Tec kinase Itk. *J. Immunol.* 2007. **179**: 7561–7567.

43 **Koretzky, G. A., Abtahian, F. and Silverman, M. A.,** SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat. Rev. Immunol.* 2006. **6**: 67–78.

44 **Jordan, M. S., Sadler, J., Austin, J. E., Finkelstein, L. D., Singer, A. L., Schwartzberg, P. L., and Koretzky, G. A.,** Functional hierarchy of the N-terminal tyrosines of SLP-76. *J. Immunol.* 2006. **176**: 2430–2438.

45 **Clements, J. L., Yang, B., Ross-Barta, S. E., Eliason, S. L., Hrstka, R. F., Williamson, R. A., and Koretzky, G. A.,** Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. *Science* 1998. **281**: 416–419.

46 **Pivniouk, V., Tsitsikov, E., Swinton, P., Rathbun, G., Alt, F. W. and Geha, R. S.,** Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76. *Cell* 1998. **94**: 229–238.

47 **Myung, P. S., Derimanov, G. S., Jordan, M. S., Punt, J. A., Liu, Q. H., Judd, B. A., Meyers, E. E. and Sigmund, C. D.,** Differential requirement for SLP-76 domains in T cell development and function. *Immunity* 2001. **15**: 1011–1026.

48 **Kumar, L., Pivniouk, V., de la Fuente, M. A., Laouini, D. and Geha, R. S.,** Differential role of SLP-76 domains in T cell development and function. *Proc. Natl. Acad. Sci. USA* 2002. **99**: 884–889.

49 **Jordan, M. S., Smith, J. E., Burns, J. C., Austin, J. E., Nichols, K. E., Aschenbrenner, A. C. and Koretzky, G. A.,** Complementation in trans of altered thymocyte development in mice expressing mutant forms of the adaptor molecule SLP76. *Immunity* 2008. **28**: 359–369.

50 **Berg, L. J.,** Signalling through TEC kinases regulates conventional versus innate CD8(+) T-cell development. *Nat. Rev. Immunol.* 2007. **7**: 479–485.

51 **Hu, J. and August, A.,** Naive and innate memory phenotype CD4+ T cells have different requirements for active Itk for their development. *J. Immunol.* 2008. **180**: 6544–6552.

Abbreviations: **7-AAD:** 7-Amino-actinomycin D · **DN:** double-negative · **DP:** double-positive · **HSA:** heat-stable antigen · **Itk-KD:** kinase-dead version of Itk · **SP:** single-positive

Full correspondence: Dr. Wilfried Ellmeier, Division of Immunobiology, Institute of Immunology, Center for Physiology, Pathophysiology and Immunology, Medical University Vienna, Lazarettgasse 19, A-1090 Vienna, Austria
Fax: +43-1-40160-965006
e-mail: wilfried.ellmeier@meduniwien.ac.at

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3.2 The transcriptional regulator PLZF induces the development of CD44 high memory phenotype T cells

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The transcriptional regulator PLZF induces the development of CD44 high memory phenotype T cells

Julia Raberger[a], Alexandra Schebesta[a,1], Shinya Sakaguchi[a,1], Nicole Boucheron[a], K. Emelie M. Blomberg[b], Anna Berglöf[b], Thomas Kolbe[c,d], C. I. Edvard Smith[b], Thomas Rülicke[c], and Wilfried Ellmeier[a,2]

[a]Division of Immunobiology, Institute of Immunology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, A-1090 Vienna, Austria; [b]Department of Laboratory Medicine, Karolinska Institutet, Clinical Research Center, S-141 86 Huddinge, Sweden; [c]Institute of Laboratory Animal Science, Research Center Biomodels Austria, University of Veterinary Medicine Vienna, A-1210 Vienna, Austria; and [d]Department of Agrobiotechnology, University of Natural Resources and Applied Life Sciences, A-1180 Vienna, Austria

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Transcriptional pathways controlling the development of CD44^{hi} memory phenotype (MP) T cells with ''innate-like'' functions are not well understood. Here we show that the BTB (bric-a-brac, tramtrack, broad complex) domain-containing protein promyelocytic leukemia zinc finger (PLZF) is expressed in CD44^{hi}, but not in CD44^{lo}, CD4$^+$ T cells. Transgenic expression of PLZF during T cell development and in CD4$^+$ and CD8$^+$ T cells induced a T cell intrinsic program leading to an increase in peripheral CD44^{hi} MP CD4$^+$ and CD8$^+$ T cells and a corresponding decrease of naïve CD44^{lo} T cells. The MP CD4$^+$ and CD8$^+$ T cells produced IFNγ upon PMA/ionomycin stimulation, thus showing innate-like function. Changes in the naïve versus memory-like subset distribution were already evident in single-positive thymocytes, indicating PLZF-induced T cell developmental alterations. In addition, CD1d-restricted natural killer T cells in PLZF transgenic mice showed impaired development and were severely reduced in the periphery. Finally, after anti-CD3/CD28 stimulation, CD4$^+$ transgenic T cells showed reduced IL-2 and IFNγ production but increased IL-4 secretion as a result of enhanced IL-4 production of the CD44^{hi}CD62L$^+$ subset. Our data indicate that PLZF is a novel regulator of the development of CD44^{hi} MP T cells with a characteristic partial innate-like phenotype.

innate-like lymphocytes | T cell development | transgenics

Peripheral CD4$^+$ and CD8$^+$ T cell subsets have been traditionally divided into naïve CD44^{lo}CD62L$^+$ and memory CD44^{hi}CD62L$^-$ populations, while the surface expression phenotype of the latter population also resembles recently activated T cells (1, 2). In recent years it became clear that the memory T cell subset is not a population consisting only of true antigen-specific memory cells that developed in response to a foreign antigen. Rather, the memory population contains a variety of different T lymphocyte subsets: some may have acquired their memory marker phenotype through homeostatic proliferation, whereas others show immediate effector function and may play a role in the front-line defense against certain bacterial infections. These additional cells were described as memory phenotype (MP) T lymphocytes, and some subsets of the MP population are also described as innate T cells (3, 4). Certain MP or innate-like T cells are derived from double-positive (DP) thymocytes, and at least some of these cells can be selected on non-classical MHC class Ib molecules (5), achieved by interaction with hematopoietic cells rather than with thymic epithelial cells (6). Among the innate-like T lymphocyte subsets that have been characterized are natural killer T cells (NKT cells) (7–9), H2-M3 specific T cells (10), mucosal-associated invariant T cells (11), and also CD8$\alpha\alpha$ expressing intraepithelial lymphocytes of the gut (12).

Important insight about signaling molecules that are involved in conventional versus innate-like T cell differentiation is provided from studies on Tec family kinase-deficient mice (4). IL2-inducible T-cell kinase (Itk) and resting lymphocyte kinase (Rlk) are essential for the development of conventional CD8$^+$ T cells. As a consequence, *Itk*$-/-$ mice and *Itk*$-/-$*Rlk*$-/-$ mice are almost devoid of conventional CD8$^+$ T cells, whereas the remaining CD8$^+$ T cell subsets have several characteristics of innate-like T lymphocytes (13–16). Conventional CD4$^+$ T cells are also reduced in the absence of Itk; thus, *Itk*$-/-$ mice have also a relative increase in innate-like MP CD4$^+$ T cells (17). In addition, *Itk*$-/-$ mice have reduced numbers of NKT cells (18–20). Another important signaling cascade that has been shown to differentially influence conventional and innate-like MP T lymphocyte development involves the signaling lymphocyte activation molecule and signaling lymphocyte activation molecule-associated protein (21–24).

To gain more insight into the function of Itk in T cells, Affymetrix microarray experiments were performed to identify genes that are differentially expressed between WT and *Itk*$-/-$ T cells (K.E.M.B., unpublished work). One of the genes identified that was up-regulated in *Itk*$-/-$ CD3$^+$ T cells was *Zbtb16*, which encodes for the transcriptional repressor promyelocytic leukemia zinc finger (PLZF). PLZF regulates several biological processes and has also been implicated in tumorigenesis (25). PLZF belongs to the family of BTB (bric-a-brac, tramtrack, broad complex) domain-containing zinc finger factors (BTB-ZF) (25, 26), and important functions in the T cell lineage have been described for several members of the BTB-ZF gene family (27).

In this study we investigated the role of PLZF in T cells. We show that PLZF was expressed in the CD4$^+$CD44^{hi} T cell lineage but not in the naïve CD4$^+$CD44^{lo} subset, whereas CD8$^+$ T cells did not express detectable levels of PLZF. Enforced transgenic expression of PLZF in T lymphocytes induced a T cell intrinsic program leading to an increase in CD44^{hi} MP T cells and a corresponding decrease of naïve CD44^{lo} T cells. The CD44^{hi} population produced IFNγ upon PMA/ionomycin stimulation, thus sharing characteristics with innate T lymphocytes. Transgenic CD4$^+$ T cells showed enhanced IL-4 levels after anti-CD3/CD28 stimulation and an increase in a CD44^{hi}CD62L$^+$ IL-4-producing subset. Together, our data indicate that PLZF is a novel transcriptional regulator for the development of CD44^{hi} MP T cells with innate-like features.

Results

Expression of PLZF Is Restricted to CD4$^+$CD44^{hi} T Cell Subsets. We have performed Affymetrix microarray analysis to identify genes

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[1]A.S. and S.S. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: wilfried.ellmeier@meduniwien.ac.at.

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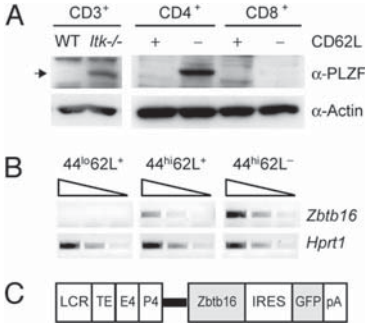

Fig. 1. PLZF expression in peripheral T cells is restricted to CD4$^+$CD44^{hi} T cells (*A*) Protein expression levels of PLZF in WT and *Itk*−/− peripheral CD3$^+$ T cells (*Left*), and in WT peripheral CD4 and CD8 T cell subsets that were sorted into CD62L$^+$ and CD62L$^-$ populations (*Right*). The equivalent of 1.5 × 10^6 cells was loaded per lane. Actin was used as loading control. Data are representative of two different experiments. (*B*) Semiquantitative RT-PCR analysis shows *Zbtb16* expression in various WT peripheral CD4$^+$ T cell subsets. *Hprt1* expression was used as loading control. The triangle indicates fivefold dilutions of input. Data are representative of two independent experiments. (*C*) Schematic map of the transgenic construct used for generation of *Zbtb16* transgenic mice. The thick bar indicates splicing module from the *Cd4* locus. (LCR/TE, locus control region and thymocyte enhancer from the *Cd4* locus; E4, proximal *Cd4* enhancer; P4, *Cd4* promoter.)

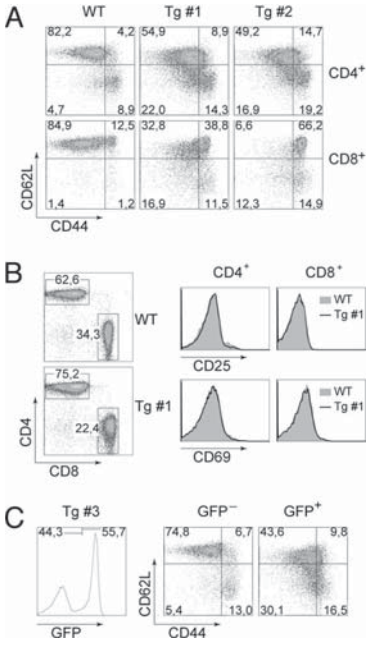

Fig. 2. Forced expression of PLZF induces a T cell intrinsic CD44^{hi} phenotype in CD4$^+$ and CD8$^+$ T cells. (*A*) CD62L and CD44 expression pattern on CD4$^+$ (*Upper*) and CD8$^+$ (*Lower*) splenic T cells from WT and PLZF transgenic mice (lines 1 and 2). Numbers in the dot plots indicate the percentage of cells in the respective quadrant. Data are representative of at least three independent experiments for each transgenic line. (*B*) CD69 and CD25 expression on CD4$^+$ and CD8$^+$ splenic T cells isolated from WT and PLZF transgenic mice. CD3-gated dot plots indicate gating regions for the histogram analysis. Data are representative of at least three independent experiments. (*C*) CD62L and CD44 expression pattern on GFP$^-$ (*Left*) or GFP$^+$ (*Right*) CD4$^+$ T cells isolated from the spleen of female PLZF mice (line 3). Numbers indicate the percentage of cells in the respective dot plot quadrants. Gating areas for GFP$^-$ and GFP$^+$ populations are shown in the CD3$^+$ T cells histogram on the left. Data are representative of five independent experiments.

that are differentially expressed between WT and *Itk*−/− CD3$^+$ T lymphocytes during T cell activation (K.E.M.B., unpublished work). One gene identified that was already expressed at higher levels in unstimulated Itk-deficient CD3$^+$ T cells compared with WT cells was *Zbtb16*, which encodes for the transcriptional regulator PLZF. Immunoblot analysis confirmed the up-regulation of PLZF in *Itk*−/− T cells (Fig. 1*A*). Because T cell subsets in *Itk*−/− mice have a T cell memory phenotype (13–17), we investigated whether the increase in the expression of PLZF was caused by a higher percentage of MP T cells in *Itk*−/− mice. Therefore, WT CD4$^+$ and CD8$^+$ T cell subsets were sorted into CD62L$^+$ and CD62L$^-$ fractions. Immunoblot analysis of these sorted subsets revealed that PLZF was expressed in the WT CD4$^+$CD62L$^-$ T cell population but in neither CD4$^+$CD62L$^+$ nor CD8$^+$ T cells (Fig. 1*A*). The majority of WT cells within the CD62L$^+$ subset are naïve CD44^{lo} T cells, and there is only a minor fraction of CD44^{hi}CD62L$^+$ cells. To test whether the CD44^{hi}CD62L$^+$ population expressed *Zbtb16*, RT-PCR analysis using RNA from sorted CD44^{lo}CD62L$^+$, CD44^{hi}CD62L$^+$, and CD44^{hi}CD62L$^-$ CD4$^+$ T cell subsets was performed. In agreement with the protein expression data, the RT-PCR analysis revealed expression of *Zbtb16* in CD44^{hi}CD62L$^-$ cells (Fig. 1*B*). In addition, low-level expression of *Zbtb16* was observed in CD44^{hi}CD62L$^+$ cells, in contrast to CD44^{lo}CD62L$^+$ cells (Fig. 1*B*). Together, this set of data indicated a correlation between PLZF expression and the CD44^{hi} MP CD4$^+$ T cell subset. To investigate a potential role of PLZF in the generation and/or function of the CD4$^+$CD44^{hi} T cell subset, gain-of-function experiments were performed. Therefore, transgenic mice expressing PLZF under the control of *Cd4 cis*-regulatory element were generated (Fig. 1*C*), which direct expression from the DN3 stage on, and remain active in, CD4$^+$ and CD8$^+$ T cell subsets (data not shown). Further, the transgenic expression vector contained an *IRES-GFP* cassette to detect transgenic T cells by GFP expression. Three different founder lines (lines 1, 2, and 3) were selected for further studies. In thymocytes, PLZF was expressed at higher levels in line 2 compared with line 1, correlating with GFP levels [supporting information (SI) Fig. S1]. Furthermore, transgenic peripheral CD4$^+$ T cells showed high levels of PLZF protein compared with WT MP CD4$^+$CD62L$^-$ T cells, although PLZF levels were only slightly enhanced in line 2 compared with line 1 (Fig. S1).

Transgenic Expression of PLZF Induces Dose-Dependent Changes in CD44 and CD62L Expression on Peripheral T Cells. PLZF transgenic mice displayed a moderate alteration in peripheral T cell numbers and had increased CD4/CD8 ratios (Fig. S2 *A* and *B*). Because the PLZF expression pattern suggested a specific function of PLZF in the CD44^{hi} T cell subset, the CD44 versus CD62L expression pattern on peripheral splenic and LN T cells was determined in PLZF transgenic mice. This analysis revealed a decrease in the CD44^{lo}CD62L$^+$ naïve CD4$^+$ and CD8$^+$ T cell subset and a corresponding increase of the CD44^{hi}CD62L$^-$ population in the presence of PLZF (Fig. 2*A* and Fig. S3). We also noted an increase in the CD44^{lo}CD62L$^-$ population in PLZF transgenic mice compared with WT mice. Furthermore, the percentage of CD44^{hi}CD62L$^+$ as well as CD44^{hi}CD62L$^-$ cells increased with increasing levels of PLZF expression, indicating a dose-dependent effect of PLZF (Fig. 2*A* and Fig. S2*C*). Transgenic CD4$^+$ and CD8$^+$ T cells showed no up-regulation of CD25 and CD69 activation marker expression (Fig. 2*B*), indicating that the presence of CD44^{hi} T cell population is not the result of T cell activation.

PLZF Induces a T Cell Intrinsic Program that Leads to a Change in the CD44 and CD62L T Cell Subset Distribution. Another transgenic founder line (line 3) had an X-chromosomal transgene insertion.

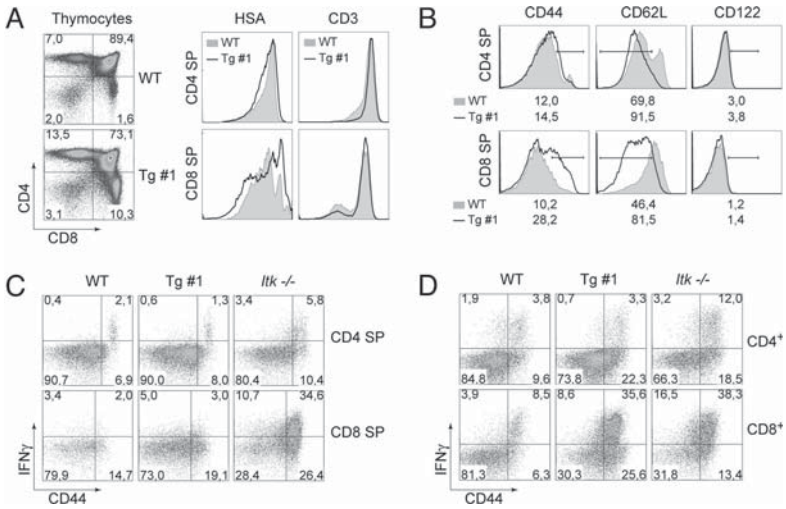

Fig. 3. MP T cells in PLZF transgenic mice develop in the thymus and have innate-like characteristics. (*A*) CD4 and CD8 expression on WT and PLZF transgenic (line 1) thymocytes. Histograms (*Right*) show HSA and CD3 expression levels on CD4SP and CD8SP cells. (*B*) CD44, CD62L, and CD122 expression pattern on CD4SP (*Upper*) and CD8SP (*Lower*) thymocytes. Numbers below histograms indicate the percentage of cells in the indicated region. Data in *A* and *B* are representative of at least three mice. (*C* and *D*) Intracellular IFNγ expression in *ex vivo* PMA/ionomycin-stimulated WT, PLZF transgenic (line 1), and *Itk*$-/-$ CD4SP and CD8SP thymocytes (*C*) and CD4$^+$ and CD8$^+$ splenic T cells (*D*). Numbers in the dot plots indicate the percentage of cells in the respective quadrants. Data in C and D are representative of three experiments.

Transgenic males of this line transmitted the mutation exclusively to their female offspring. Furthermore, almost all T cells of male mice of line 3 carrying the PLZF transgene were GFP$^+$ (data not shown), whereas in hemizygous female mice of line 3, approximately only half of the T cells were GFP$^+$ (Fig. 2*C*). Because there were similar numbers of GFP$^+$ and GFP$^-$ T cells in female transgenic mice, we concluded that PLZF-expressing T cells have no major developmental advantage or disadvantage over non-transgenic T cells. Similar to transgenic lines 1 and 2, GFP$^+$ CD4$^+$ and CD8$^+$ T cells in male and female mice of line 3 displayed the altered CD44/CD62L expression pattern in the spleen (Fig. 2*C* and data not shown), whereas the GFP$^-$ population in female transgenic mice showed a CD44/CD62L distribution similar to WT mice (Fig. 2*C*). Thus, PLZF induces a T cell-intrinsic genetic program in both CD4$^+$ and CD8$^+$ T cells that leads to a dramatic alteration in the CD44/CD62L subset distribution.

CD44^{hi} T cells in PLZF Transgenic Mice Develop in the Thymus and Have a Memory Phenotype with Innate-Like Characteristics. To determine whether the CD44^{hi} T cells emerge already in the thymus, a comprehensive analysis of thymocyte development was performed. In contrast to WT cells, transgenic PLZF thymocytes had increased percentages of SP cells and reduced numbers of DP cells (Fig. 3*A* and Fig. S4*A*). However, the expression of CD3 and HSA on the various subsets was similar between WT and PLZF thymocytes (Fig. 3*A*). Furthermore, CD4SP and CD8SP PLZF transgenic thymocytes showed a larger fraction of cells that had down-regulated CD62L expression compared with WT SP cells, and PLZF transgenic CD8SP cells displayed an increase in CD44^{hi} subsets (Fig. 3*B*). CD122 expression was similar in WT and PLZF transgenic SP cells (Fig. 3*B*). In agreement with the thymic emergence of PLZF MP T cells, T cells with an altered CD44/CD62L profile were already present in 14-day-old PLZF transgenic mice (Fig. S5).

The CD44^{hi} MP T cell population of WT mice is composed of several T cell subsets (3, 4). To test whether CD44^{hi} SP cells and peripheral T cells in PLZF transgenic mice have immediate effector function, thymocytes and splenocytes were isolated and stimulated *ex vivo* with PMA/ionomycin. In addition, we compared the innate properties of PLZF transgenic T cells with *Itk*$-/-$ SP thymocytes and splenocytes, as *Itk*$-/-$ mice have a large number of innate-like T cells (13–16). Similar to the WT CD4SP and CD8SP CD44^{hi} populations, CD4SP and CD8SP CD44^{hi} PLZF transgenic T cells produced IFNγ, although the percentage of IFNγ-producing CD4SP cells was reduced within the CD44^{hi} fraction (Fig. 3*C* and Fig. S4*B*). *Itk*$-/-$ SP thymocytes, as reported previously (13, 14), showed increased production of IFNγ compared with WT cells. In the periphery, the percentage of IFNγ-producing CD4$^+$ T cells within the CD44^{hi} population was reduced in PLZF transgenic mice compared with WT controls (Fig. 3*D* and Fig. S4*C*). In contrast, there was an increase in IFNγ-producing CD44^{hi} CD8$^+$ T cells. The relative percentage of PLZF transgenic IFNγ-producing CD8$^+$ T cells within the CD44^{hi} population was moderately increased compared with WT MP T cells, but not as high as in innate-like *Itk*$-/-$ CD8$^+$ T cells (Fig. 3*D* and Fig. S4*C*). Thus, despite the dramatic increase in the numbers of MP T cells in PLZF transgenic mice, the PLZF MP population had similar innate-like functions as WT MP subsets.

Many innate T lymphocytes such as NKT cells (7) and innate-like CD8$^+$ T cells in *Itk*$-/-$ mice require IL-15 for their survival (13, 15). IL-15 signals via the IL-15 receptor complex, which is composed of several subunits including CD122 (28). PLZF transgenic and WT CD44^{hi}CD62L$^+$ CD8$^+$ T cells displayed similar up-regulated CD122 expression levels, and the percentage of CD122$^+$ cells within this transgenic subset was even slightly increased compared with the WT subset (Fig. S6). Together, these data indicate that enforced expression of PLZF leads to the development of CD44^{hi} MP T cells with innate-like characteristics.

Impaired NKT Cell Development in PLZF Transgenic Mice. Because NKT cells have a CD44^{hi}CD62L$^-$ surface expression phenotype (7), we investigated whether PLZF expression led to an increase in NKT cells. To have internal staining controls, the analysis was performed in female PLZF transgenic mice of line 3, which have both GFP$^+$ and GFP$^-$ cells within the T cell subset. CD1d

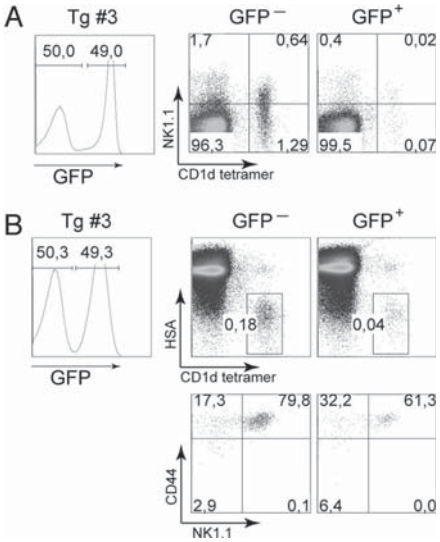

Fig. 4. Impaired NKT cell development in PLZF transgenic mice. (*A*) NK1.1 expression and CD1d-tet binding on GFP$^-$ (*Left*) and GFP$^+$ (*Right*) CD3$^+$ transgenic splenocytes (line 3). Numbers in the dot plots indicate the percentage of cells in the respective quadrant. Histogram shows gating region for GFP$^-$ and GFP$^+$ T cell populations. Data shown are representative of three mice. Similar results have also been obtained for line 1 (n = 3 mice). (*B*) Histogram (*Upper*) shows gating region for GFP$^-$ and GFP$^+$ thymocytes (line 3). Dot plots indicate gating regions for CD1d-tet$^+$ HSA^{lo} NKT cells. CD44 and NK1.1 expression on CD1d-tet$^+$ HSA^{lo} NKT cells (*Lower*). Numbers in the dot plots indicate the percentage of cells in the respective quadrant. Data shown are representative of three mice. Similar results have also been obtained for line 1 (n = 3).

tetramers (CD1d-tet) loaded with the αGalCer analogue PBS57 were used to detect invariant NKT cells. This analysis revealed that CD1d-tet$^+$ NKT cells were severely reduced in the GFP$^+$ T cell subset, whereas NKT cells were readily detected within the GFP$^-$ subset (Fig. 4*A* and Fig. S7*A*). Next, we investigated whether the peripheral reduction of NKT cells is caused by a (partial) block during thymic NKT cell development. Early CD1d-tet$^+$ NKT cells are HSA^{hi}, followed by a HSA^{lo} stage (7, 8). Preliminary results indicated that the GFP$^+$/GFP$^-$ ratio was approximately 50:50 in the HSA^{hi}CD1d-tet$^+$ fraction, whereas the percentage of GFP$^+$ cells declined in the HSA^{lo}CD1d-tet$^+$ fraction (data not shown). This indicated a defect in the transition to or within the HSA^{lo} fraction. The thymic CD1d-tet$^+$ HSA^{lo} subset can be further divided into three stages that follow a CD44$^-$NK1.1$^-$ (stage 1), CD44^{hi}NK1.1$^-$ (stage 2), and CD44^{hi}NK1.1$^+$ (stage 3) pattern (7, 8). Our analysis revealed that the transition from stage 2 to stage 3 is impaired in PLZF transgenic mice, indicated by an increase in stage 2 and a corresponding decrease in stage 3 NKT cells (Fig. 4*B* and Fig. S7*B*). This suggests that enforced expression of PLZF impairs NKT cell development.

Transgenic Expression of PLZF Alters the Cytokine Profile of Naïve (CD44^{lo}) and MP (CD44^{hi}) CD4$^+$ T Cells. Next we analyzed whether PLZF expression changed the cytokine production of CD4$^+$ T cells upon T cell receptor-mediated activation. Total CD4$^+$ T cells were isolated from WT or PLZF transgenic mice and stimulated with anti-CD3/CD28. IL-2 levels were severely impaired in PLZF transgenic CD4$^+$ T cells (Fig. 5*A*), and PLZF transgenic CD4$^+$ T cells produced reduced amounts of IFNγ compared with WT CD4$^+$ T cells. In contrast, they showed dramatically increased levels of IL-4 compared with WT controls (Fig. 5*A*).

To investigate which subset is responsible for the dramatic increase in IL-4, CD44^{lo}CD62L$^+$, CD44^{hi}CD62L$^+$, and CD44^{hi}CD62L$^-$, WT and PLZF transgenic CD4$^+$ T cells were sorted and activated with anti-CD3/CD28. All subsets of WT and PLZF transgenic CD4$^+$ T cells produced IL-2, whereas IFNγ was secreted by CD44^{hi}CD62L$^+$ and CD44^{hi}CD62L$^-$ T cells (Fig. 5*B*). However, transgenic T cells showed reduced IL-2 and IFNγ production compared with WT cells. WT CD44^{hi}CD62L$^-$ cells produced high levels of IL-4, whereas IL-4 levels in this subset of transgenic T cells were reduced (Fig. 5*B*). This is in contrast to the CD44^{hi}CD62L$^+$ subset, which showed enhanced IL-4 levels in PLZF transgenic mice. Thus, the presence of this IL-4 producing CD44^{hi}CD62L$^+$ subset explains why total CD4$^+$ T cells produce elevated levels of IL-4. Although *Tbx21* (encoding for T-bet) and *Gata3* are known to be major transcription factors responsible for IFNγ and IL-4 expression of Th1 and Th2 cells, respectively, no major difference in the expression of *Tbx21* and *Gata3* in the various subsets was observed (Fig. S8).

Discussion

In this study we showed that the BTB-ZF factor PLZF is predominantly expressed in CD44^{hi} CD4$^+$ T cells. Enforced expression of PLZF during T cell development and in peripheral T cells led to the appearance of a large population of peripheral T cells with a CD44^{hi} memory phenotype that were able to produce IFNγ upon *ex vivo* PMA/ionomycin stimulation. PLZF expressing (i.e., GFP$^+$) T cells in female mice of line 3 showed an MP despite the presence of similar numbers of GFP$^-$ T cells with a naïve phenotype, thus indicating a T cell intrinsic defect. Together with the observations that the cells were not activated and that PLZF T cells showed no increased BrdU incorporation (data not shown), this also suggests that the CD44^{hi} T cells are not generated via homeostatic proliferation (29). In addition,

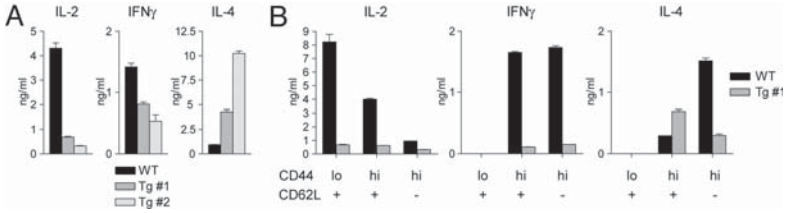

Fig. 5. PLZF alters the cytokine profile of T cell receptor-stimulated CD4$^+$CD44^{lo} and CD4$^+$CD44^{hi} T cells. (*A*) Diagrams show IL-2, IFNγ, and IL4 production in purified WT and PLZF transgenic (lines 1 and 2) CD4$^+$ T cells stimulated for 48 h with plate-bound anti-CD3/CD28. For IFNγ detection, only cells with >95% purity were used to exclude ''contaminating'' IFNγ-producing CD8$^+$ T cells. The cytokine levels in the supernatants were determined by ELISA in duplicates. Data shown are representative of three independent experiments. (*B*) IL-2, IFNγ, and IL-4 production of WT or PLZF transgenic (line 1) CD4$^+$ T cells that were sorted into CD44^{lo}CD62L$^+$, CD44^{hi}CD62L$^+$, and CD44^{hi}CD62L$^-$ subsets. Cells were stimulated and cytokines determined as in *A*. Data shown are representative of four independent experiments (three with line 1 and one with line 3; male mice).

changes in the naïve versus memory-like subset distribution were already evident at the SP stage during T cell development. This indicates that the MP T cell populations in PLZF transgenic mice are at least in part derived from DP thymocytes, like other MP T cells (3, 4). We therefore propose that PLZF induces a transcriptional program that leads to the generation of $CD44^{hi}$ MP T cells that share certain features with innate-like T cells.

The observations that PLZF is up-regulated in the *Itk*−/− T cell population (which is enriched in innate-like T cells) and that PLZF expression is restricted to the $CD4^+CD44^{hi}$ population revealed a correlation between PLZF expression and MP $CD4^+$ T cells. A more direct link between PLZF and innate-like MP T cells is established by the $CD44^{hi}$ phenotype of transgenic T cells. Thus, ectopic PLZF expression leads to an increase of those T cell subsets that express PLZF in WT cells, indicating that PLZF can convert $CD4^+CD44^{lo}$ subsets into $CD4^+CD44^{hi}$ populations. Furthermore, endogenous PLZF was expressed in transgenic $CD44^{hi}$ but not $CD44^{lo}$ $CD4^+$ T cells (data not shown), an expression pattern identical to the one in WT $CD4^+$ T cell subsets. This indicates that transgenic PLZF induced the differentiation of a distinct lineage of MP T cells with innate-like characteristics. Interestingly, immunoblot analysis did not reveal expression of PLZF in innate-like $CD8^+$ T cells. However, PLZF also induced the $CD44^{hi}$ phenotype in $CD8^+$ T cells, suggesting that PLZF may induce similar transcriptional programs in CD4 and CD8 T cell lineages, and possibly indicating the existence of an endogenous factor that specifically directs the development of $CD44^{hi}$ MP $CD8^+$ T cells.

We demonstrated that $CD4^+$ and $CD8^+$ MP T cells in PLZF mice possess innate-like characteristics. The increase in IFNγ-producing transgenic $CD44^{hi}$ $CD8^+$ T cells is similar to the one observed in innate-like *Itk*−/− $CD8^+$ T cells, although the percentage of IFNγ-positive cells within the $CD44^{hi}$ population is higher in the absence of Itk. Further, only peripheral MP $CD8^+$ T cells and not CD8SP MP thymocytes in PLZF transgenic mice showed an increase in IFNγ-producing $CD44^{hi}$ cells upon PMA/ionomycin stimulation, suggesting a further maturation of innate-like functions in the periphery. In contrast, IFNγ-producing $CD44^{hi}$ cells were increased in the thymus and in the periphery in *Itk*−/− mice. These findings may indicate a different subset composition and/or differential regulation of innate-like $CD8^+$ T cells in PLZF and *Itk*−/− mice. PLZF MP $CD4^+$ T cells produced IFNγ as well; however, the percentage of IFNγ-positive $CD4^+$ T cells within the $CD44^{hi}$ population was reduced. Moreover, upon anti-CD3/CD28 activation we observed reduced cytokine production in all subsets of PLZF transgenic $CD4^+$ T cells compared with WT $CD4^+$ T cell subsets, except for the dramatic increase of IL-4 production in the $CD44^{hi}CD62L^+$ subset. It is possible that PLZF may downmodulate the IL-2 and IFNγ response by binding to cytokine loci and recruiting co-repressors including nuclear receptor corepressor and histone deacetylase (25), or by repressing the expression of an important signaling molecule or transcription factor required for cytokine expression. Alternatively, but not mutually exclusively, PLZF expression may lead to a different cellular composition of peripheral $CD4^+$ T cells, even though we could not observe any altered expression of T-bet and Gata-3 in the various subsets of PLZF transgenic mice compared with WT mice. A "candidate" innate-like population are NKT cells that share a $CD44^{hi}$ expression phenotype with PLZF transgenic T cells (7–9). A recent study has shown that PLZF is primarily expressed in CD1d-restricted NKT cells (30). However, other MP T cell subsets may express PLZF as well, as PLZF expression was up-regulated in *Itk*−/− T cells, which have diminished numbers of NKT cell subsets compared with WT T cells (19, 20). Remarkably, NKT cell numbers in mice lacking PLZF were severely reduced, and they also displayed impaired cytokine expression patterns (30). Unexpected given this essential role of PLZF for NKT cell development, we observed a severe reduction of NKT cells in PLZF expressing mice compared with WT mice. One explanation, that both PLZF-deficient and PLZF transgenic mice have diminished numbers of NKT cells, may come from the observation that stage 1 and stage 2 NKT cells express the highest level of PLZF and down-regulate PLZF expression at stage 3 (30). Thus, enforced transgenic expression of PLZF may partially block NKT cells at this stage.

Taken together, our study gives insight into transcriptional control mechanisms that regulate conventional versus MP T cell development and suggest that PLZF is an important regulator in this process. Further studies aiming to identify and characterize the genetic program that is induced by PLZF are required to further elucidate these developmental processes.

Materials and Methods

Generation of the PLZF Transgenic Construct. The transgene expression cassette consisted of the *Cd4* Locus control region/thymocyte enhancer, the *Cd4* proximal enhancer/promoter, and a splicing module from silencer-less *Cd4* intron 1 sequences, followed by *IRES-GFP-polyA* sequences, and was provided by Ichiro Taniuchi (Riken, Yokohama, Japan). The cDNA sequence encoding for PLZF (MGI: 103222; nucleotide 249-2352) was inserted as an EcoRI fragment upstream of the *IRES-GFP* module.

Generation of PLZF Transgenic Mice. The linearized construct was microinjected into a pronucleus of fertilized C57BL/6N inbred oozytes that were transferred afterward into the oviduct of pseudopregnant foster mothers according to standard protocols for generating transgenic mice (31). Transgene integrations were identified by PCR of tail DNA with GFP-specific primers. Of several identified transgenic founders, we selected lines C57BL/6N-*Tg(Cd4-Zbtb16,GFP)141–143 Biat* (lines 1–3 for brevity) based on the GFP expression levels.

Mice. Animals used in this study were bred and maintained in the animal facility of the Medical University of Vienna. Analyzed mice were 6 to 8 weeks of age unless indicated otherwise. All animal experiments were approved by the Federal Ministry for Science and Research.

Flow Cytometric Analysis and Antibodies. The following antibodies were used for the staining: PE-anti-CD62L, PE-anti-CD122, PE-Cy7-anti-CD4, PE-anti-IFNγ, and APC-anti-IL-4 from BD Pharmingen; APC-anti-CD8α (53–6.7), APC-anti-CD62L, PE-Cy7-anti-CD44, APC-anti-CD25, APC-anti-NK1.1, PE-anti-CD3e, and Pb-anti-CD3e from eBioscience; and A647-anti-CD3 from Caltag. PBS57-loaded and unloaded CD1d tetramers (conjugated with PE) were obtained from the National Institutes of Health tetramer facility and used according to the instructions. Flow cytometry was performed on LSRII (BD Biosciences) and data analyzed using FlowJo software.

Purification of T Cells and Cell Sorting. $CD3^+$ T cells were isolated by incubating cell suspensions of spleens (in PBS solution supplemented with 2% FCS) with biotinylated anti-CD11b, anti-CD11c, anti-CD45R, anti-Ly-6G, anti-Ter119, and anti-NK antibodies, followed by negative depletion using streptavidin beads (BD Pharmingen) according to the manufacturer's instructions. For the isolation of $CD4^+$ T cells, biotinylated anti-CD8α antibodies were added to the depletion antibody mixture. The purity of either $CD3^+$ or $CD4^+$ T cells was assessed by flow cytometry and was routinely >90%–95%. For cell sorting, $CD4^+$ T cells were incubated with anti-CD4, anti-CD44 and anti-CD62L, and $CD4^+$ T cells subsets were sorted into $CD44^{lo}CD62L^+$, $CD44^{hi}CD62L^+$, and $CD44^{hi}CD62L^-$ populations with a FACSAria cell sorter (Becton Dickinson).

Activation of T Cells and Cytokine Measurement. Purified or FACS-sorted $CD4^+$ T cells (5×10^5) or T cell subsets (5×10^5) were stimulated with plate-bound anti-CD3ε (1 μg/ml) and plate-bound anti-CD28 (3 μg/ml) on 48-well plates. $CD4^+$ T cells were grown in 1 ml RPMI1640 Glutamax-I (Invitrogen), supplemented with 10% FCS (Invitrogen), antibiotics, and 2-mercaptoethanol (Invitrogen). The supernatants for cytokine quantification were collected 48 h later. IL-2, IL-4, and IFNγ cytokine levels were determined by ELISA (BD Pharmingen).

For PMA/ionomycin stimulation, thymocytes or splenocytes were incubated for 5 h with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma) in the presence of GolgiSTOP (BD Biosciences). Surface staining for CD3, CD4, CD8, and CD44 was performed, and subsequently cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen) and further stained with PE-anti-IFNγ.

Statistical Analysis. All data are expressed as mean ± SEM. Statistical analysis was performed by using a Student *t* test. The *P* values were defined as following: **P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; n.s., not significant.

Note Added in Proof. While this article was in revision, Savage *et al.* (32) reported similar data as described in our study. They observed that transgenic expression of PLZF induced CD4SP thymocytes and CD4+ T cells to acquire a $CD44^{hi}$ phenotype. In addition, Savage *et al.* reported that PLZF is primarily expressed in CD1d-restricted NKT cells and that PLZF-deficient mice have impaired NKT cell differentiation and effector function (in agreement with ref. 30).

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1. Swain SL, *et al.* (2006) CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. *Immunol Rev* 211:8–22.
2. Prlic M, Williams MA, Bevan MJ (2007) Requirements for CD8 T-cell priming, memory generation and maintenance. *Curr Opin Immunol* 19:315–319.
3. Surh CD, Boyman O, Purton JF, Sprent J (2006) Homeostasis of memory T cells. *Immunol Rev* 211:154–163.
4. Berg LJ (2007) Signalling through TEC kinases regulates conventional versus innate CD8(+) T-cell development. *Nat Rev Immunol* 7:479–485.
5. Rodgers JR, Cook RG (2005) MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* 5:459–471.
6. Urdahl KB, Sun JC, Bevan MJ (2002) Positive selection of MHC class Ib-restricted CD8(+) T cells on hematopoietic cells. *Nat Immunol* 3:772–779.
7. Bendelac A, Savage PB, Teyton L (2007) The biology of NKT cells. *Annu Rev Immunol* 25:297–336.
8. Kronenberg M, Engel I (2007) On the road: progress in finding the unique pathway of invariant NKT cell differentiation. *Curr Opin Immunol* 19:186–193.
9. MacDonald HR, Mycko MP (2007) Development and selection of Valpha l4i NKT cells. *Curr Top Microbiol Immunol* 314:195–212.
10. Colmone A, Wang CR (2006) H2–M3-restricted T cell response to infection. *Microbes Infect* 8:2277–2283.
11. Treiner E, Lantz O (2006) CD1d- and MR1-restricted invariant T cells: of mice and men. *Curr Opin Immunol* 18:519–526.
12. Lambolez F, Kronenberg M, Cheroutre H (2007) Thymic differentiation of TCR alpha beta(+) CD8 alpha alpha(+) IELs. *Immunol Rev* 215:178–188.
13. Atherly LO, *et al.* (2006) The Tec family tyrosine kinases Itk and Rlk regulate the development of conventional CD8+ T cells. *Immunity* 25:79–91.
14. Broussard C, *et al.* (2006) Altered development of CD8+ T cell lineages in mice deficient for the Tec kinases Itk and Rlk. *Immunity* 25:93–104.
15. Dubois S, Waldmann TA, Müller JR (2006) ITK and IL-15 support two distinct subsets of CD8+ T cells. *Proc Natl Acad Sci USA* 103:12075–12080.
16. Hu J, Sahu N, Walsh E, August A (2007) Memory phenotype CD8+ T cells with innate function selectively develop in the absence of active Itk. *Eur* J Immunol 37:2892–2899.
17. Hu J, August A (2008) Naive and Innate Memory Phenotype CD4+ T Cells Have Different Requirements for Active Itk for Their Development. *J Immunol* 180:6544–6552.
18. Gadue P, Stein PL (2002) NK T cell precursors exhibit differential cytokine regulation and require Itk for efficient maturation. *J Immunol* 169:2397–2406.
19. Au-Yeung BB, Fowell DJ (2007) A key role for Itk in both IFN gamma and IL-4 production by NKT cells. *J Immunol* 179:111–119.
20. Felices M, Berg LJ (2008) The tec kinases itk and rlk regulate NKT cell maturation, cytokine production, and survival. *J Immunol* 180:3007–3018.
21. Veillette A, Dong Z, Latour S (2007) Consequence of the SLAM-SAP signaling pathway in innate-like and conventional lymphocytes. *Immunity* 27:698–710.
22. Griewank K, *et al.* (2007) Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* 27:751–762.
23. Li W, *et al.* (2007) The SLAM-associated protein signaling pathway is required for development of CD4+ T cells selected by homotypic thymocyte interaction. *Immunity* 27:763–774.
24. Horai R, *et al.* (2007) Requirements for selection of conventional and innate T lymphocyte lineages. *Immunity* 27:775–785.
25. Kelly KF, Daniel JM (2006) POZ for effect−POZ-ZF transcription factors in cancer and development. *Trends Cell Biol* 16:578–587.
26. Collins T, Stone JR, Williams AJ (2001) All in the family: the BTB/POZ, KRAB, and SCAN domains. *Mol Cell Biol* 21:3609–3615.
27. Bilic I, Ellmeier W (2007) The role of BTB domain-containing zinc finger proteins in T cell development and function. *Immunol Lett* 108:1–9.
28. Ma A, Koka R, Burkett P (2006) Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu Rev Immunol* 24:657–679.
29. Stockinger B, Kassiotis G, Bourgeois C (2004) Homeostasis and T cell regulation. *Curr Opin Immunol* 16:775–779.
30. Kovalovsky D, *et al.* (2008) The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol*, 10.1038/ni.1641.
31. Rulicke T (2004) Pronuclear microinjection of mouse zygotes. *Methods Mol Biol* 254:165–194.
32. Savage AK, *et al.* (2008) The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29:391–403.

Supporting Information

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SI Methods

Immunoblotting. Purified cells ($5 \times 10^6/100\ \mu l$) were lysed in Carin lysis buffer (20 mM Tris-HCl pH 8.0, 138 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% Nonidet P-40, 10% glycerol, 2 mM NA-vanadate) supplemented with complete protease inhibitors (Roche). Proteins were then separated on 8% SDS-polyacrylamide gels and blotted onto PVDF membranes (Bio-Rad). Anti-PLZF antibody (mAb 2A9) was purchased from Calbiochem. Anti-actin was from Sigma and secondary antibodies were from Jackson Immunoresearch.

cDNA Synthesis and RT-PCR. Total RNA was isolated from sorted peripheral $CD4^+$ T cell subsets using TRI reagent (Sigma). cDNA synthesis was performed using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Cycling conditions for RT-PCR were 4 min at 95°C, followed by 25 to 35 cycles of 30 sec at 95°C, 30 sec at 58°C, and 40 sec at 72°C. The primer sequences were: *Hprt1*, F 5′-ATT GTG GCC CTC TGT GTG CT; R 5′-TTG CGC TCA TCT TAG GCT TTG; *Zbtb16*, F 5′-CCC AGT GGA GAA GCA TTT GG; R 5′-ACC GTT TTC CGC AGA GTT CA. For the detection of endogenous *Zbtb16*, the sequence was F 5′-TGG CTG TGG CAA GAA GGT CA; R 5′-AAC CAA GGA GGG CAG AGA CC.

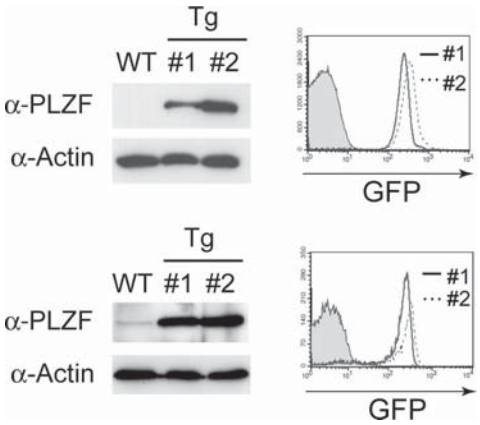

Fig. S1. Immunoblot analysis showing PLZF expression levels in thymocytes (*Upper*) and CD4$^+$ T cells (*Lower*) isolated from transgenic animals of lines 1 and 2. Actin was used as loading control. The equivalent of 8×10^5 thymocytes and 1.5×10^6 CD4$^+$ T cells was loaded per lane. For WT CD4$^+$, T cells were sorted for CD62L$^-$ subsets. Histogram (*Right*) depicts GFP expression levels in transgenic thymocytes (*Upper*) and CD4$^+$ T cells (*Lower*) (Line 1, thick line; line 2, dotted line.) The shaded area indicates a WT control. All data are representative of two independent experiments.

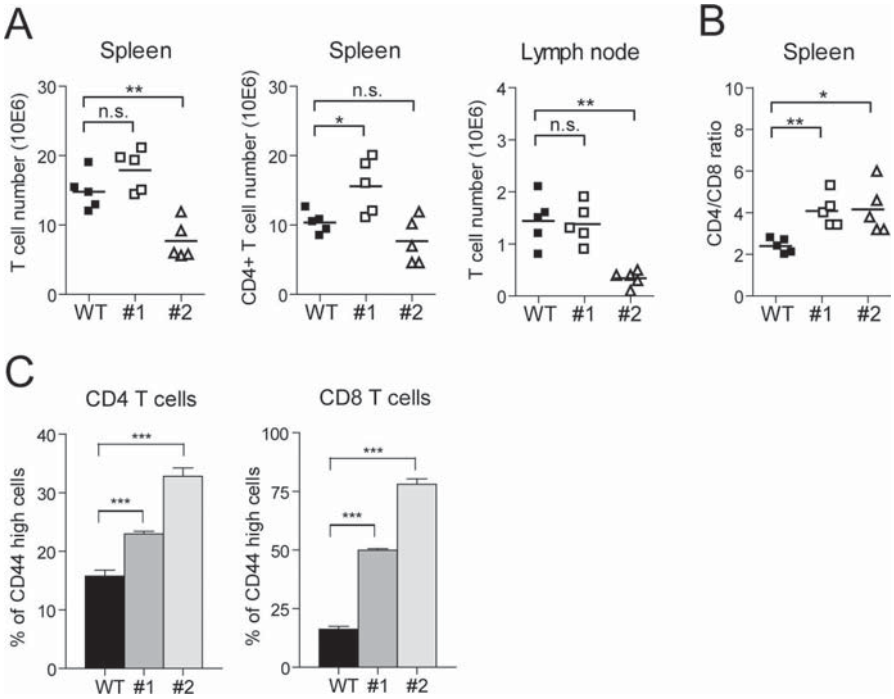

Fig. S2. (*A*) Numbers of splenic CD3$^+$ (*Left*) and CD4$^+$ T cells (*Middle*), and of lymph node CD3$^+$ T cells (*Right*) in either WT mice or in animals of PLZF transgenic lines 1 and 2. Each symbol represents one mouse. (*B*) Diagram shows the splenic CD4/CD8 T cell ratio of WT mice, and of transgenic lines 1 and 2 mice. Each symbol represents one mouse. (*C*) The percentage of CD44^{hi} cells in splenic CD4$^+$ and CD8$^+$ T cell subsets is WT and PLZF transgenic mice (lines 1 and 2) is shown ($n = 4$).

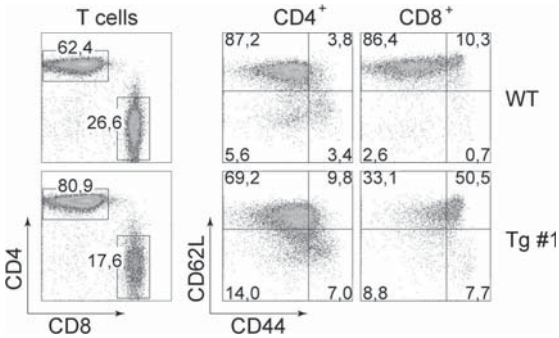

Fig. S3. CD4 and CD8 expression pattern on LN CD3$^+$ T cells isolated from WT mice and from PLZF transgenic line 1 (*Left*). CD62L and CD44 expression pattern on CD4$^+$ and CD8$^+$ gated T cells (*Right*). Numbers in the dot plots indicate the percentage of cells in the respective quadrant. Data are representative of at least six independent experiments for each transgenic line.

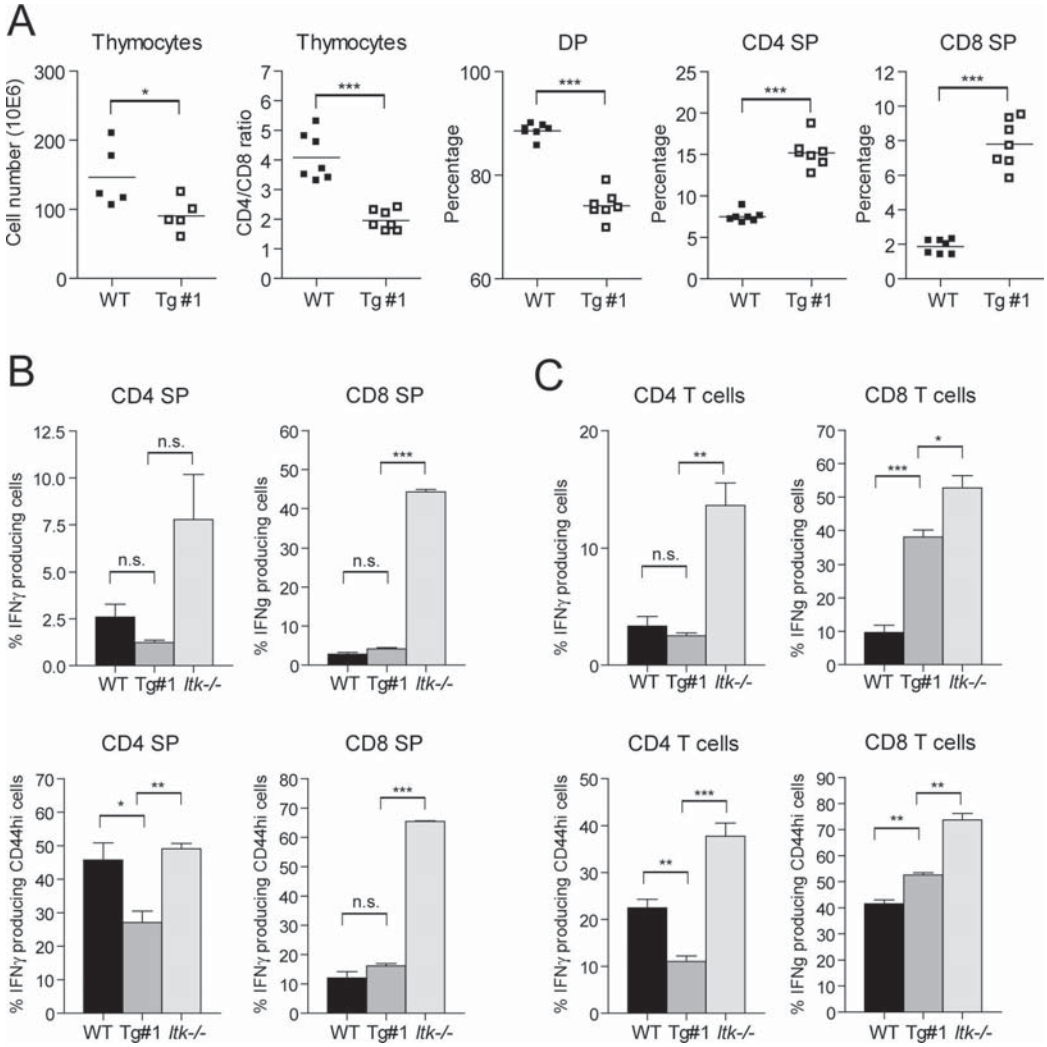

Fig. S4. (*A*) Statistical analysis of WT and PLZF transgenic (line 1) thymocytes. Diagrams show total thymocyte numbers (*Left*), the thymic CD4SP/CD8SP ratio (second from left), and the percentage of DP, CD4SP, and CD8SP thymocytes (third, fourth, and fifth panels, respectively). Each symbol represents one mouse. (*B* and *C*) Summary showing IFNγ production of *ex vivo* PMA/ionomycin stimulated thymocytes (*B*) and splenocytes (*C*). Percentage of IFNγ-producing CD4SP and CD8SP thymocytes (*Upper*) and percentage of IFNγ-producing cells within the CD44^{hi} population of CD4SP and CD8SP cells (*Lower*). In *C*, splenocytes were gated on CD4$^+$ or CD8$^+$ T cells. Results shown are the summary of three mice analyzed.

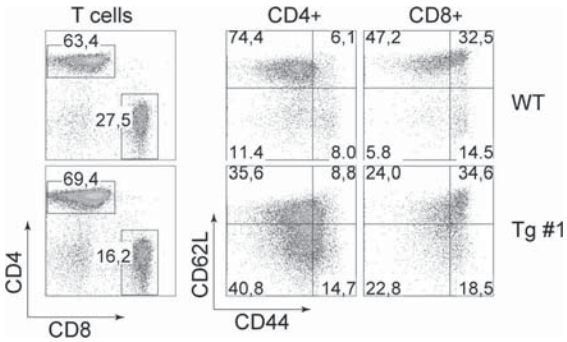

Fig. S5. CD62L and CD44 expression on splenic $CD4^+$ and $CD8^+$ T cells isolated from 2-week-old WT and PLZF transgenic mice (line 1). Dot plot (*Left*) indicates gating regions for $CD4^+$ and $CD8^+$ T cells. Data are representative of four mice.

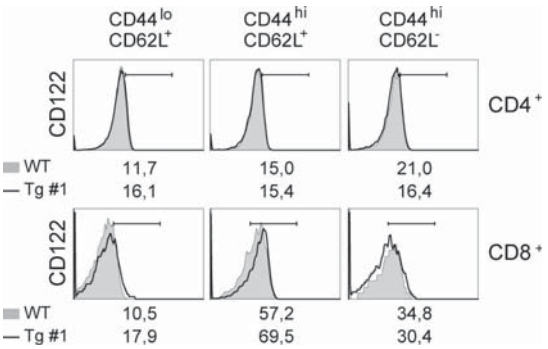

Fig. S6. CD122 expression in PLZF transgenic (line 1) $CD4^+$ (*Upper*) and $CD8^+$ (*Lower*) $CD44^{lo}CD62L^+$, $CD44^{hi}CD62L^+$, and $CD44^{hi}CD62L^-$ T cell subsets. Numbers below histograms indicate the percentage of cells in the indicated region. Data are representative of three mice.

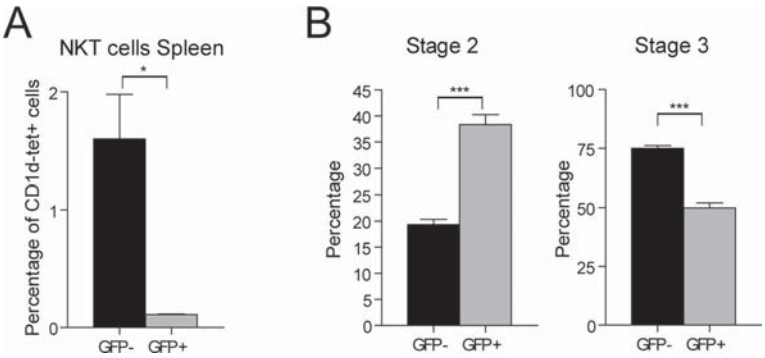

Fig. S7. (A) The percentage of GFP$^-$ and GFP$^+$ CD4$^+$ transgenic CD1d-tet$^+$ NKT cells from the spleen is shown. The results are the summary of three mice analyzed. (*B*) Statistical analysis of the thymic NKT cell defect in WT and PLZF transgenic mice (line 3). Diagrams indicate the percentage of stage 2 (CD44$^+$NK1.1$^-$) and stage 3 (CD44$^+$NK1.1$^+$) CD1d-tet$^+$HSA^{lo} NKT cells of all mice analyzed ($n = 3$).

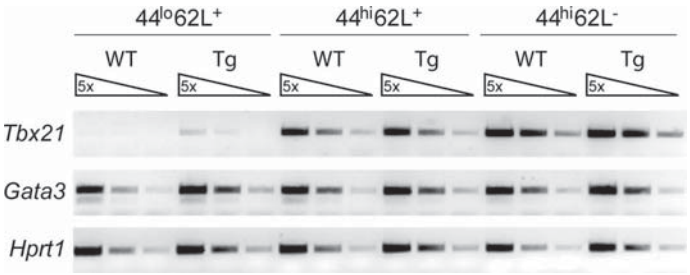

Fig. S8. Semiquantitative RT-PCR analysis shows *Tbx21* (encoding for T-bet) and *Gata3* expression in various peripheral CD4$^+$ T cell subsets. *Hprt1* expression was used as loading control. Data are representative of two different experiments (line 3). The following primers were used: *Tbx21*, F 5′-TGC AGT GTG GAA AGG CAG AA; R 5′-GGG TAG AAA CGG CTG GGA AC; *Gata3*, F 5′-CAA TGC CTG CGG ACT CTA CC; R 5′-GTC AGC ATG TGG CTG GAG TG.

3.3 The protein tyrosine kinase Tec regulates IL-17-producing memory-like CD4$^+$ T cells

Summary

IL-17 expression has to be tightly controlled during an immune response. Here we report that an IL-17-producing CD44^{hi}CD62L$^-$ memory-like CD4$^+$ T cell subset is regulated by the protein tyrosine kinase Tec. Despite normal levels of IFNγ and IL-4, *Tec–/–* Th1 and Th2 cultures as well as activated CD4$^+$ T cells produced increased amount of IL-17, and this correlated with elevated expression of IL-23R by these cells. However, Tec was not required for Th17 cell differentiation *in vitro*. Directly *ex vivo* stimulated *Tec–/–* CD4$^+$ T cells contained an increased population of CD44^{hi} memory-like CD4$^+$ T cells that produced IL-17, and Tec expression was strongly up-regulated in memory-like CD4$^+$ T cells upon TCR stimulation. Furthermore, activated *Tec–/–* memory-like CD4$^+$ T cells produced higher levels of IL-17 and showed increased STAT3 phosphorylation upon IL-23 stimulation. Thus, our data indicate a critical role for Tec in signaling pathways that regulate the generation and function of an IL-17 producing memory-like CD4+ T cell subset.

Introduction

Members of the Tec kinase family (Tec, Btk, Itk, Rlk and Bmx) form the second largest class of protein tyrosine kinases that are activated during lymphocyte development and activation. T cells express three members of the Tec kinase family, Tec, Itk and Rlk. A large number of bio-chemical studies and the analysis of mice that are single- and double-deficient for Itk and Rlk have identified several processes in which this kinase family plays important roles [1-4]. *Itk–/–* mice have defects in T cell development and function [5,6]. Positive selection of CD4$^+$ T cells is impaired in the absence of Itk and it was suggested that Tec family kinases modulate signaling thresholds during T cell development [7]. TCR signaling in naïve T cells and therefore activation and proliferation is impaired in the absence of Itk, and *Itk–/–* T cells were unable to polarize towards a Th2 phenotype, even if primed under Th2-polarizing conditions [8,9]. Itk was described to negatively regulate expression of T-bet, thus providing a molecular mechanism of how Th2 differentiation is impaired in the absence of Itk [10]. Itk was also shown to be involved in activation of β1 integrins by the TCR [11] and in the regulation of the cytoskeleton [12-14]. Much less is known about the role of

Rlk in T cells. *Rlk–/–* mice had no major developmental alteration and only a mild signaling defect within the T cell lineage [15]. The analysis of *Itk–/–Rlk–/–* T cells however showed that Rlk is involved in TCR-mediated signaling events and that it partially compensates for the loss of Itk, indicated by a further diminished proliferative response upon anti-CD3 stimulation and a decrease in IL-2 and IFNγ production in *Itk–/–Rlk–/–* T cells compared to *Itk–/–* T cells [15]. Further, *Itk–/–Rlk–/–* mice showed an increased susceptibility to an intracellular pathogen Toxoplasma gondii [15]. Taken together, these studies showed that Itk and also Rlk play an important role in T cell development and activation, and in effector T cell differentiation.

To address the role of Tec in lymphocytes, we used previously generated *Tec–/–* mice [16]. The initial analysis of *Tec–/–* animals primarily focused on B cells, which developed and functioned normally in the absence of Tec. However, the combined deletion of Tec and Btk resulted in a severe impairment of B lymphocyte development and function [16], indicating that in the B cell lineage Tec compensates for loss of Btk. With respect to the T cell lineage, much less is known about the role of Tec. *In vitro* studies using a murine T cell hybridoma line have shown that Tec is activated in response to TCR/CD28 stimulation [17]. It has also been shown that CD28 engagement leads to recruitment of Tec to the cytoplasmic tail of CD28 and overexpression of Tec activates both IL-2 and IL-4 promoters [17,18]. Recently, it was reported that Tec expression is up-regulated in primary T cells suggesting a function of Tec in effector T cells [19]. Nevertheless, the function of Tec in T lymphocytes is poorly understood.

In this study we performed a comprehensive analysis of the helper T cell lineages in *Tec–/–* mice. Tec function in $CD4^+$ T cells was dispensable for TCR-mediated signaling events, Ca^{2+} flux and proliferation. However, despite normal levels of IFNγ and IL-4, *Tec–/–* Th1 and Th2 cultures as well as activated $CD4^+$ T cells displayed increased levels of IL-17. This correlated with increased *Il23* receptor (*Il23r*) expression in activated $CD4^+$ T cells. Tec was not required for TGFβ plus IL-6 mediated Th17 differentiation. *Ex vivo* PMA/ionomycin-stimulated *Tec–/–* $CD4^+$ T cells contained an increased population of $CD44^{hi}$ memory-like $CD4^+$ T cells that produced IL-17, and Tec expression was strongly up-regulated in memory-like $CD4^+$ T cells upon TCR stimulation. Moreover, *Tec–/–* memory-like $CD4^+$ T cells showed increased STAT3 phosphorylation upon IL-23 stimulation and produced higher levels of IL-17 compared to wildtype (WT) memory-like $CD4^+$ T cells upon activation. Our data link Tec family kinases with memory-like $CD4^+$ T cell function and indicate that Tec is a regulator of IL-17-producing memory-like $CD4^+$ T cells.

Results

Normal T cell proliferation and TCR signaling in *Tec–/–* $CD4^+$ T cells

Tec–/– mice were generated to investigate the role of Tec in the hematopoietic system [16]. *Tec–/–* mice have normal thymocyte numbers and a normal CD4/CD8 subset distribution that is indistinguishable from WT thymocytes [16]. Peripheral T cell numbers are normal and there is no alteration in the ratio of naïve ($CD44^{lo}CD62L^+$) to memory-like ($CD44^{hi}CD62L^-$) $CD4^+$ and $CD8^+$ T cell subsets (Figure 1A and data not shown). Furthermore, $CD4^+CD25^+$ regulatory T cells are present at similar numbers in WT and *Tec–/–* mice (Figure 1B).

To test whether Tec is required for efficient T cell proliferation, $CD4^+$ T cells from WT and *Tec–/–* mice were isolated and stimulated with various concentrations of plate-bound anti-CD3. There was no difference in the proliferative capacity of *Tec–/–* $CD4^+$ T cells compared to WT $CD4^+$ T cells (Figure 1C). This correlated with a similar up-regulation of the early activation markers CD25 and CD69 (data not shown). Several studies have implicated Tec in the CD28 co-stimulatory pathway and in the production of IL-2 [18,21,22]. However, proliferation in response to anti-CD28-mediated co-stimulation and IL-2 production was similar in *Tec–/–* and WT $CD4^+$ T cells (Figure 1D). This is in agreement with previous data showing a normal anti-CD28 co-stimulatory response of *Tec–/–* $CD3^+$ T cells [16].

TCR crosslinking leads to the tyrosine phosphorylation and activation of several proteins [23]. Therefore, we investigated the phosphorylation pattern and kinetics by immunoblot analysis using the pY-specific antibody 4G10. The overall tyrosine phosphorylation patterns were similar in WT and *Tec–/–* T cells (Figure 1E), indicating normal activation upon TCR stimulation. Ca^{2+}-flux was normal in *Tec–/–* $CD4^+$ T cells (Figure 1F), and in agreement with this data PLC□1 Y783 phosphorylation, which has been shown to be critical for PLCγ1 activity in T cells [24] was not significantly impaired (data not shown). Additionally, there were no differences in the activation of the MAP kinases Erk1/2 (data not shown).

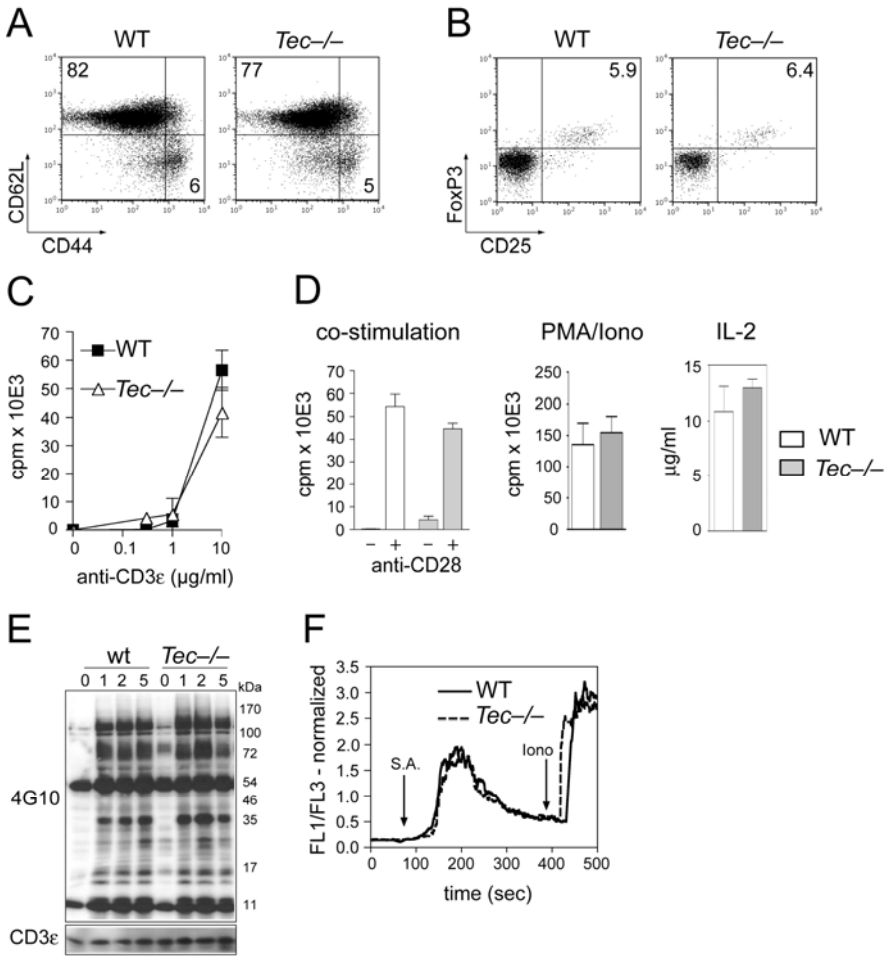

Figure 1. Tec is not essential during the primary activation of CD4$^+$ T cells. (A) CD44 and CD62L expression on WT and *Tec–/–* splenocytes. Cells were gated on CD4$^+$ spleen cells. Numbers in the dotplot indicate the percentage of cells in the respective quadrant. Data are representative of 6 mice. (B) Dot plots show CD25 and intracellular FoxP3 expression of WT and *Tec–/–* splenocytes. The cells were gated on CD4$^+$ spleen cells. Numbers in the quadrant indicate the percentage of cells in the respective quadrant. Data are representative of 6 mice. (C) CD4$^+$ T cells were isolated from mice of the indicated genotype and stimulated for 60 hours with the indicated amount of plate-bound anti-CD3. Proliferation was assessed in triplicates by [^{3}H]thymidine incorporation during the last 16 hours. Data are representative of 4 independen t experiments. (D) CD4$^+$ T cells were stimulated for 60 hours with plate-bound anti-CD3 (0.3 µg/ml) ± plate-bound anti-CD28 (3 µg/ml) (left panel) or PMA (50 ng/ml) and ionomycin (500 ng/ml) (middle panel). Proliferation was assessed in triplicates by [^{3}H]thymidine incorporation during the last 16 hours. Right panel: Bar diagram showing IL-2 levels in the culture supernatants after 42 hours stimulation with anti-CD3/CD28. One representative of 4 independent experiments is shown. (E) Purified CD3$^+$ T cells from WT and *Tec–/–* mice were incubated for 1 min with biotinylated anti-CD3 (1 µg/ml) and fully activated for the indicated time points with streptavidin. The phosphorylation pattern and kinetic of signaling components in the cell lysates were assessed by western blot using the anti-phospho Y-specific antibody 4G10. Equal loading was controlled with anti-CD3ε. Data are representative of 4 independent experiments. (F) Purified CD4$^+$ T cells were labelled with Fluo-4 and Fura Red. T cells were incubated for 1 min with biotinylated anti-CD3 (1 µg/ml) and fully activated by crosslinking with streptavidin. Ca^{2+} flux is represented over time as a normalized ratio between Fluo-4 and Fura Red emission. Abbreviations: S. A.: streptavidin, Iono: ionomycin. One representative experiment out of 2 is shown.

IL-17 levels in *Tec–/–* Th1 and Th2 cells are increased

Several studies have shown that the Tec family kinase Itk plays a major role in the differentiation of naïve T cells into Th2 helper T cells [4]. The observation that Tec expression is up-regulated in primary T cells and in Th1 and Th2 cells suggested a function of Tec in effector T cells [19]. To investigate this in more detail, WT and *Tec–/–* $CD4^+$ T cells were purified and stimulated *in vitro* under the appropriate Th1- or Th2-polarizing conditions. *Tec–/–* Th1 and Th2 cells showed no difference in IFNγ and IL-4 levels, respectively, compared to WT cells (Figure 2A and 2B). However, Tec is required in Th1 and Th2 cells for high-level production of IL-2 (Figure 2B). Surprisingly, we also noted increased levels of IL-17 in the supernatants of Th1 and Th2 cultures (Figure 2B). Supernatants of activated *Tec–/–* $CD4^+$ T cells that were kept under non-polarizing conditions show a similar increase in IL-17 secretion (Figure 2B). To determine which subset produced IL-17 in the polarized cultures, intracellular levels of IL-17 were measured. This revealed that a distinct subset of IL-17-producing T cells was present in Th1 and Th2 cultures and these cells did not produce IFNγ or IL-4, respectively (Figure 2C).

IL-17 producing $CD4^+$ T cells represent a recently characterized subset of helper T cells, designated as Th17 cells [25-30]. It has been reported that IL-23 expands Th17 cells via IL-23 receptor (IL-23R)-mediated activation of STAT3 [31-34]. Therefore, we determined whether there is a difference in IL-23R expression levels between WT and *Tec–/–* cells. $CD4^+$ T cells were activated with anti-CD3/CD28 under non-polarizing conditions and restimulated at day 6 with anti-CD3. Under these conditions, *Tec–/–* $CD4^+$ T cells displayed much higher expression levels of *Il23r* than WT cells (Figure 3A) and produced also higher levels of IL-17 (Figure 3B). Next, we investigated whether the increase in IL-17 and the elevated expression of the *Il23r* in *Tec–/–* $CD4^+$ T cells correlated with increased STAT3 phosphorylation. In contrast to WT $CD4^+$ T cells, which showed low levels of phosphorylated STAT3 (pSTAT3), *Tec–/–* $CD4^+$ T cells displayed much higher levels of pSTAT3 (Figure 3C).

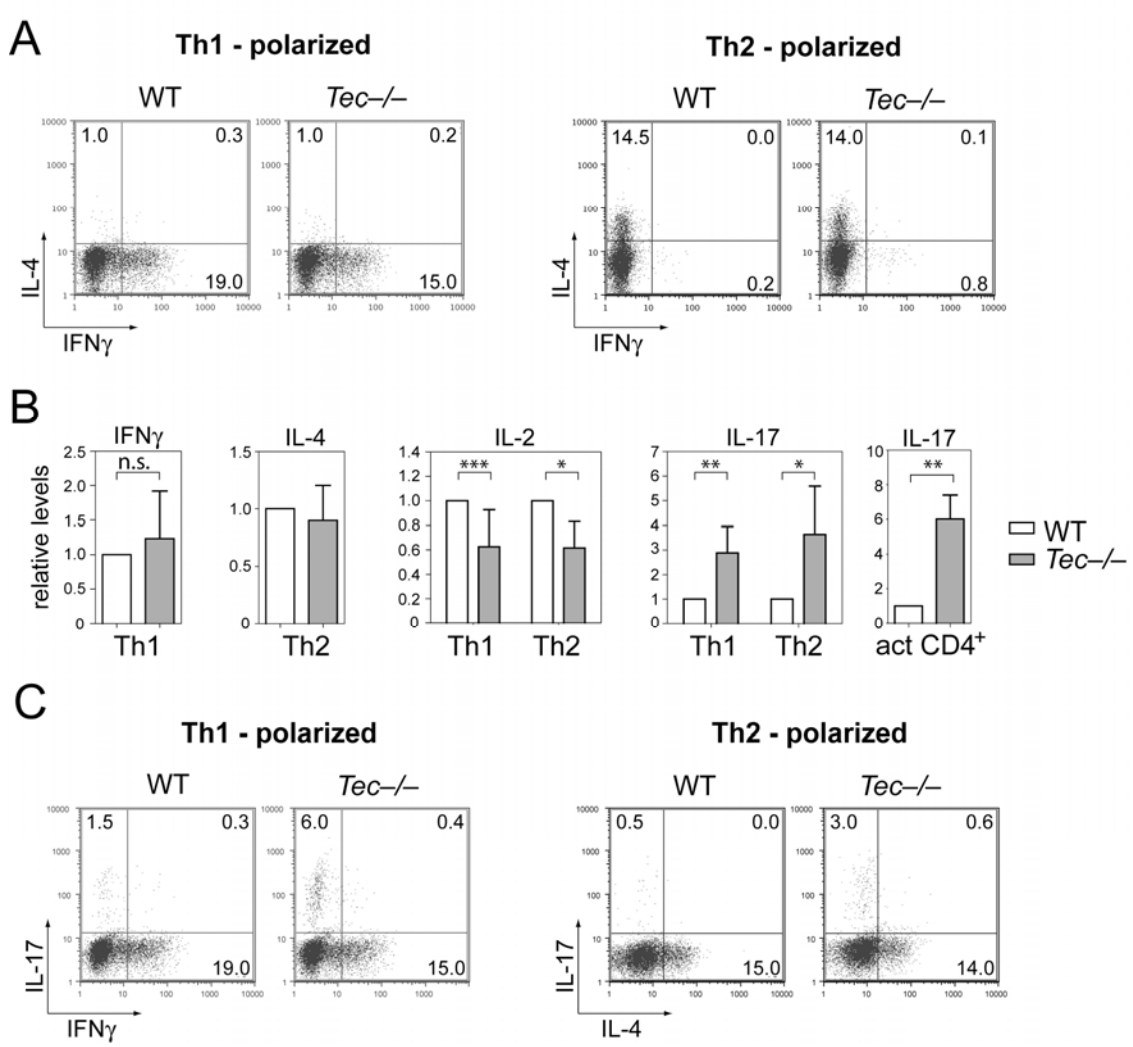

Figure 2. Cytokine expression patterns in Th1 and Th2 polarized WT and *Tec–/–* CD4$^+$ T cells. (A) Purified CD4$^+$ T cells of the indicated genotype were activated with plate-bound anti-CD3 (1 µg/ml) plus plate-bound anti-CD28 (3 µg/ml) and cultured for 5 days under Th1 or Th2 polarization conditions. Dead cells were removed using a lymphoprep gradient, and purified polarized cells were restimulated with anti-CD3 (0.1 µg/ml) for 12 h. Dotplots show intracellular IL-4 and IFNγ expression in Th1 (left panel) and Th2 (right panel) polarized cells. One representative of four independent experiments is shown. (B) Bar diagrams showing cytokine levels in the supernatant of Th1 and Th2-polarized cells, and in non-polarized activated CD4$^+$ T cells (act CD4$^+$) as measured by ELISA. WT expression levels of the respective cytokine in each individual experiment was set as one and the expression levels in *Tec–/–* CD4$^+$ T cells relative to wt cells was calculated. The concentration range for the various cytokines in the different experiments for WT cells was 1.5-10 ng/ml for IFNγ, 0.9-2 ng/ml for IL-4, 2-7 ng/ml (Th1) and 0.2-1.5 ng/ml (Th2) for IL-2, 0.3-0.5 ng/ml (Th1), 0.5-1 ng/ml (Th2) and 0.05-0.2 ng/ml (act. CD4+) for IL-17. Data shown are the summary of several independent experiments: n=4 for IFNγ and IL-4, n=3 for IL-2 and IL-17. (C) CD4$^+$ T cells were purified and treated as in (A). Dotplots show intracellular expression of IL-17 and IFNγ in Th1 (left panel) and IL-17 and IL-4 in Th2 (right panel) polarized cells. One representative of two independent experiments is shown.

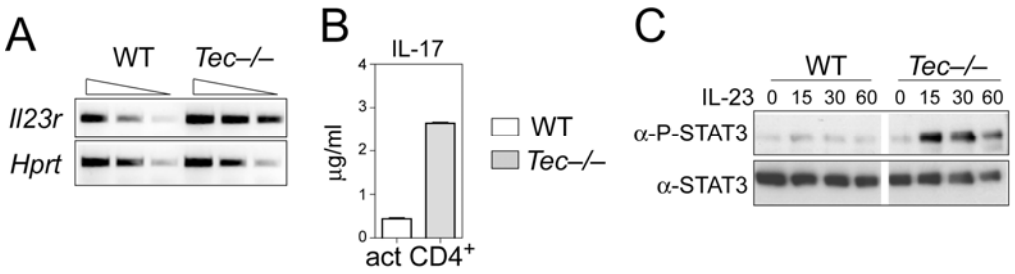

Figure 3. Increased *Il23* receptor expression and STAT3 phosphorylation in *Tec–/–* CD4$^+$ T cells. (A) Semi-quantitative RT-PCR analysis shows *Il23r* expression in WT and *Tec–/–* CD4$^+$ T cells. *Hprt1* expression was used as loading control. RNA was isolated from CD4$^+$ T cells that were activated with plate-bound anti-CD3 (1 µg/ml) plus plate-bound anti-CD28 (3 µg/ml) for 5 days and restimulated with anti-CD3 for 8 hours. The triangle indicates 5-fold dilutions of input. Data are representative of 2 different experiments. (B) IL-17 expression levels in the culture supernatant of activated CD4$^+$ T cells that were used for the isolation of mRNA as described in (A). (C) CD4$^+$ T cells were activated as described in (A), and restimulated with IL-23 for the indicated time periods. The cell lysate equivalent of 1 x 10^6 CD4$^+$ T cells was loaded on a 10 % SDS-polyacrylamide gel and the phosphorylation status (Y705) and expression of STAT3 was assessed by immunoblot analysis. Data are representative of 3 independent experiments. (A-C) Data shown were generated from the same batch of CD4$^+$ T cells.

Th17 differentiation and EAE induction is normal in the absence of Tec

Th17 cells are involved in many autoimmune diseases and enhance the clearance of extracellular bacteria and fungi [25-30]. Th17 cells can be generated from naïve CD4$^+$ T cells in the presence of TGFβ plus IL-6 [35-37], and mainly secrete the pro-inflammatory cytokines IL-17A (or IL-17), IL-17F [38], IL-21 [39] and IL-22 [40,41]. To test whether the increase in IL-17-producing cells is caused by altered Th17 differentiation in the absence of Tec, sorted naïve CD4$^+$ T cells were polarized in the presence of TGFβ plus IL-6. We found that WT and *Tec–/–* Th17 cells were generated at the same frequency and produced similar levels of IL-17 (Figure 4A and 4B), indicating that Tec kinase does not regulate the differentiation of Th17 cells. Furthermore, the development of FoxP3-expressing regulatory T cells, generated from naïve CD4$^+$ T cells in the presence of TGFβ was not altered in the absence of Tec (Figure4A). We also could not observe a difference between WT and *Tec–/–* mice in experimental autoimmune encephalomyelitis (EAE), an *in vivo* model for autoimmune inflammation where Th17 cells have been shown to have an important function [26,42,43]. There was no difference in the clinical score and incidence of EAE (Figure 4D) or associated weight loss between WT and *Tec–/–* mice (data not shown). Examination of the spinal cord did also not reveal any difference in the amount and/or constitution of the spinal cord lesions or demyelination (data not shown). Taken together, the *in vitro* and *in vivo* studies suggest that there is no difference in Th17 cells between WT and *Tec–/–* mice.

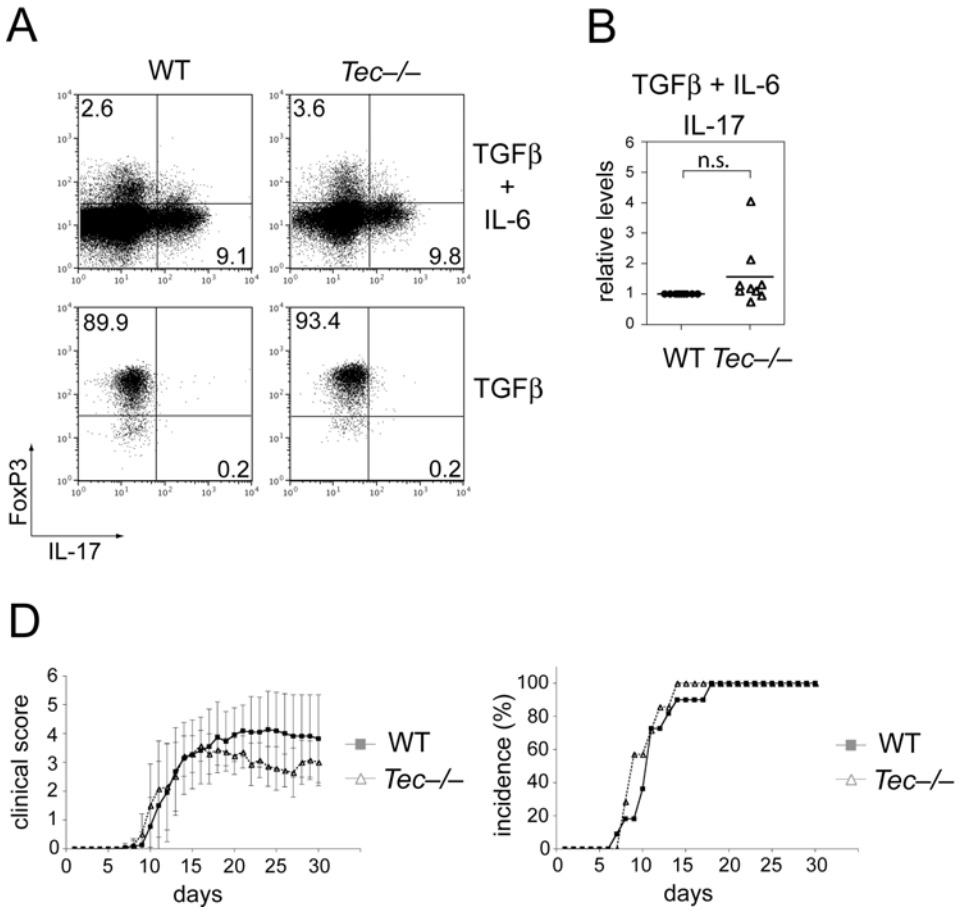

Figure 4. Normal generation of Th17 cells and EAE induction in the absence of Tec. (A) Intracellular expression of IL-17 and FoxP3 in differentiated cells that have been generated from WT and *Tec–/–* naïve $CD4^+$ T cells. Sorted $CD4^+$ T cells of the indicated genotype were activated with plate-bound anti-CD3 (1 µg/ml) plus plate-bound anti-CD28 (3 µg/ml) and cultured for 4 days with TGFβ ± IL-6. Purified polarized cells were restimulated with anti-CD3 (0.1 µg/ml) for 12 h. Numbers in the dotplots indicate the percentage of cells in the respective quadrants. Data shown are representative of six independent experiments for TGFβ + IL-6 and of two independent experiments for TGFβ only. (B) Sorted naïve $CD4^+$ T cells were activated with antiCD3/CD28 as described in (A) and cultured for 5 days with TGFβ + IL-6. Purified polarized cells were restimulated with anti-CD3 (0.1 µg/ml) for 12 h. For each experiment, WT IL-17 levels were set as one, and the diagram shows relative IL-17 levels. The IL-17 levels in WT supernatants were in a range of 2-20 ng/ml. Each symbol represents one experiment with a pool of naïve $CD4^+$ T cells isolated from at least three mice. (C) Diagram showing the clinical score (left panel) and the incidence (right panel) of EAE in WT and *Tec–/–* mice. Data show summary of a group of 11 WT and *8 Tec–/–* mice. One representative of 2 independent experiments is shown.

Loss of Tec affects the memory-like but not the naïve $CD4^+$ T cell subset

The observations that TGFβ plus IL-6-generated *Tec–/–* Th17 cells produced WT levels of IL-17, and that EAE induction is similar in WT and *Tec–/–* mice indicated that Tec does not play a major role in the differentiation of Th17 cells *in vitro* and *in vivo*. Furthermore, only Th1 and Th2 cell cultures generated from purified total (i.e. both $CD44^{lo}$ and $CD44^{hi}$ subsets) but not sorted naïve

suggested that Tec may play a role rather in memory-like ($CD44^{hi}$) than in naïve $CD4^+$ T cell subsets. To test whether the increase in IL-17 production in the absence of Tec is due to alterations in memory-like subsets or due to different signals produced by naïve $CD4^+$ T cells, we performed "mixed-culture" experiments. Naïve ($CD44^{lo}CD62L^+$) WT or *Tec–/–* $CD4^+$ T cells were co-cultured with either WT or *Tec–/–* memory-like ($CD44^{hi}CD62L^-$) $CD4^+$ T cells at a ratio of 14:1 and activated under non-polarizing conditions. The "mixed-cultures" containing *Tec–/–* memory-like T cells displayed always slightly higher levels of IL-17 compared to cultures containing WT memory-like T cells, independent whether WT or *Tec–/–* naive T cells were present (Figure 5A). However, the highest levels of IL-17 were always observed when both naive and memory-like subsets were isolated from *Tec–/–* mice (Figure 5A).

Since it appeared that the presence of either WT or *Tec–/–* naïve $CD4^+$ T cells may influence the production of IL-17 (Figure 5A), we wanted to analyze whether the observed increase in STAT3 phosphorylation in total $CD4^+$ T cells (as shown in Figure 3C) was due to increased pSTAT3 in memory-like $CD4^+$ T cells. However, after activation (and the corresponding up-regulation of CD44) it is not possible to distinguish between cells that were initially naïve or memory-like T cells. Therefore, naïve $CD4^+$ T cells from $Ly5.1^+$ WT mice were co-cultured with either WT or *Tec–/–* $Ly5.2^+$ memory-like cells at a 14:1 ratio. The co-cultures were stimulated, rested, reactivated with IL-23, and the phosphorylation of STAT3 in $Ly5.2^+$ cells of the different "mixed-cultures" was assessed by intracellular pSTAT3 detection. Only a minor population of $Ly5.2^+$ memory-like cells displayed STAT3 Y705 phosphorylation, however, a higher percentage of pSTAT-3 could be detected in *Tec–/–* memory-like cells compared to WT controls (Figure 5B, right panel). In complementary experiments in which WT or *Tec–/–* $Ly5.2^+$ naïve $CD4^+$ T cells have been activated in the presence of $Ly5.1^+$ WT memory-like cells, the naïve populations did not show detectable levels of pSTAT3 (Figure 5B, left panel). In control cells that have been stimulated with IL-6, an almost complete and uniform shift of pSTAT3 could be detected both in naïve and memory-like populations (data not shown). Thus, our data indicate that the increase in pSTAT3 in *Tec–/–* T cells is due to STAT3 phosphorylation in memory-like T cells.

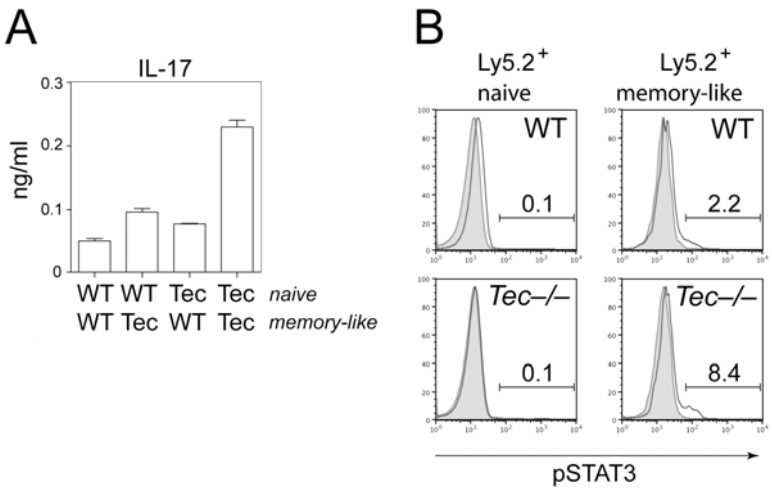

Figure 5. Memory cell populations are affected by loss of Tec. (A) WT and *Tec–/–* naïve CD4$^+$ T cells were co-cultured with WT and *Tec–/–* memory-like CD4$^+$ T cells (in a 14:1 ratio). Mixed cell subsets were activated for 5 days with plate-bound anti-CD3 (1 µg/ml) plus plate-bound anti-CD28 (3 µg/ml). 5 x 10^5 cells were restimulated with anti-CD3 for 12 hours, and IL-17 levels were determined by ELISA. Diagram shows IL-17 levels. Data shown are representative of three independent experiments. (B) Left panel: Ly5.2$^+$ WT or *Tec–/–* naive CD4$^+$ T cells were co-cultured (in a 14:1 ratio) with Ly5.1$^+$ memory-like WT CD4$^+$ T cells. Right panel: Ly5.1$^+$ naïve WT CD4$^+$ T cells were co-cultured (in a 14:1 ratio) with Ly5.2$^+$ WT or *Tec–/–* memory-like CD4$^+$ T cells. Cells were activated and purified as described in (A), and restimulated with IL-23 for 15 minutes. Histograms show STAT3 phosphorylation in naïve (left panel) and memory-like Ly5.2$^+$ (right panel) WT and *Tec–/–* subsets. Numbers in the region indicate the percentage of cells with STAT3 phosphorylation. Data are representative of two independent experiments.

Altered memory-like CD4$^+$ T cells in *Tec–/–* mice

Our data indicated that Tec might have an important function in memory-like CD4$^+$ T cells. Since it was shown that the expression of Tec was up-regulated in total effector T cells [19], suggesting a more prominent role of Tec in these cells, we investigated the expression profile of Tec in naïve and memory-like CD4$^+$ T cells. *Ex vivo* isolated memory-like CD4$^+$ T cells expressed higher levels of Tec relative to PLC compared to naïve CD4$^+$ T cells. Furthermore, Tec was dramatically up-regulated in memory-like cells upon activation (Figure 6A), suggesting an important role of Tec in this subset. To study this further, naïve and memory-like CD4$^+$ T cells were stimulated with anti-CD3/CD28, and restimulated at day 6 with anti-CD3. *Tec–/–* memory-like CD4$^+$ T cells produced higher levels of IL-17 compared to WT memory-like CD4$^+$ T cells (Figure 6B). In contrast, IL-4 and IFNγ levels were comparable between WT and *Tec–/–* memory-like CD4$^+$ T cells (Figure 6C). Since memory-like but not naïve CD4$^+$ T cells produced IL-17, these data indicate that Tec regulates an IL-17-expressing memory-like CD4$^+$ T cell subset.

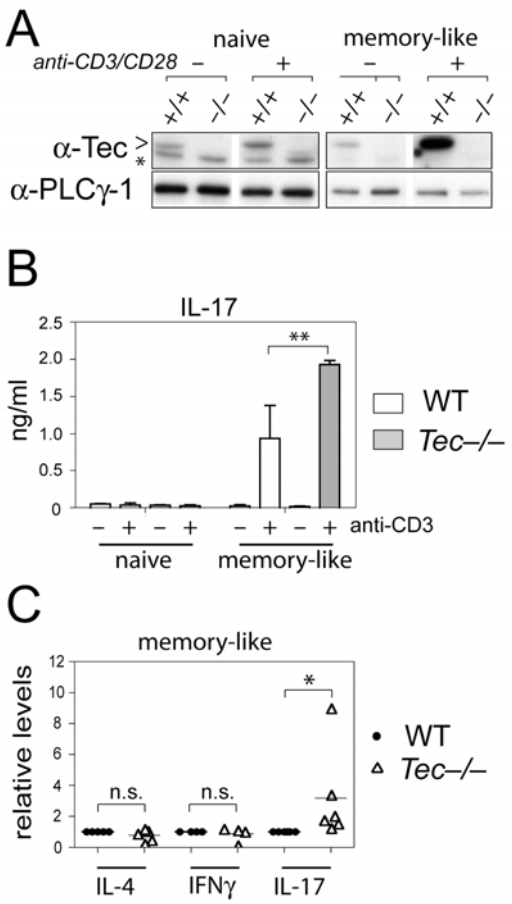

Figure 6. Tec functions in memory-like CD4$^+$ T cells. (A) Naïve (left panel) and memory (right panel) CD4$^+$ T cells of the indicated genotype were isolated and either lysed before (–) or after (+) activation for 5 days with plate-bound anti-CD3 (1 μg/ml) plus plate-bound anti-CD28 (3 μg/ml). The cell lysate equivalent of 0.5 x 10^6 CD4$^+$ T cells was loaded on a 10 % SDS-polyacrylamide gel and the expression of Tec was assessed by immunoblot analysis. PLC1 was used as loading control. > indicates the Tec-specific band, while " * " indicates a non-specific background band. One representative of two independent experiments is shown. (B) Naïve and memory-like CD4$^+$ T cells of the indicated genotype were isolated and activated for 5 days with plate-bound anti-CD3 (1 μg/ml) plus plate-bound anti-CD28 (3 μg/ml). 5 x 10^5 cells were restimulated with anti-CD3 for 12 hours. The IL-17 levels in the supernatant were determined by ELISA. One representative of two independent experiments is shown. (C) Summary showing IL-4, IFNγ and IL-17 level in memory-like CD4$^+$ T cells as determined by ELISA. Cells were activated as described in (D). For each experiment, WT cytokine levels were set as one, and the diagram shows relative cytokine levels. The cytokine levels in WT supernatants were in a range of 1.5-10 ng/ml for IL-4, 5-25 ng/ml for IFNγ, and 2-10 ng/ml for IL-17. Each symbol represents one experiment.

Memory-like T cells are not a population of CD44^{hi}CD62L$^-$ cells consisting only of true antigen-specific memory cells that developed in response to a foreign antigen. Rather, the population contains a variety of different T lymphocyte subsets and some, like true antigen-specific memory cells, have already immediate effector function [44,45]. To test whether there is already a population of *Tec–/–* CD4$^+$ T cells that has immediate effector function and produces IL-17, CD4$^+$

T cells were isolated and stimulated *ex vivo* with PMA/ionomycin. While the percentage of WT and *Tec–/–* CD44^{hi} CD4^{+} T cells that produced IFNγ or IL-4 upon PMA/ionomycin stimulation was similar (Figure 7A and 7B), there was an increase in the percentage of IL-17-producing *Tec–/–* CD44^{hi} CD4^{+} T cells compared to WT CD44^{hi} CD4^{+} T cells. This indicates that a subset of IL-17-producing CD4^{+} T cells is increased within the memory-like population in *Tec–/–* mice with immediate effector function.

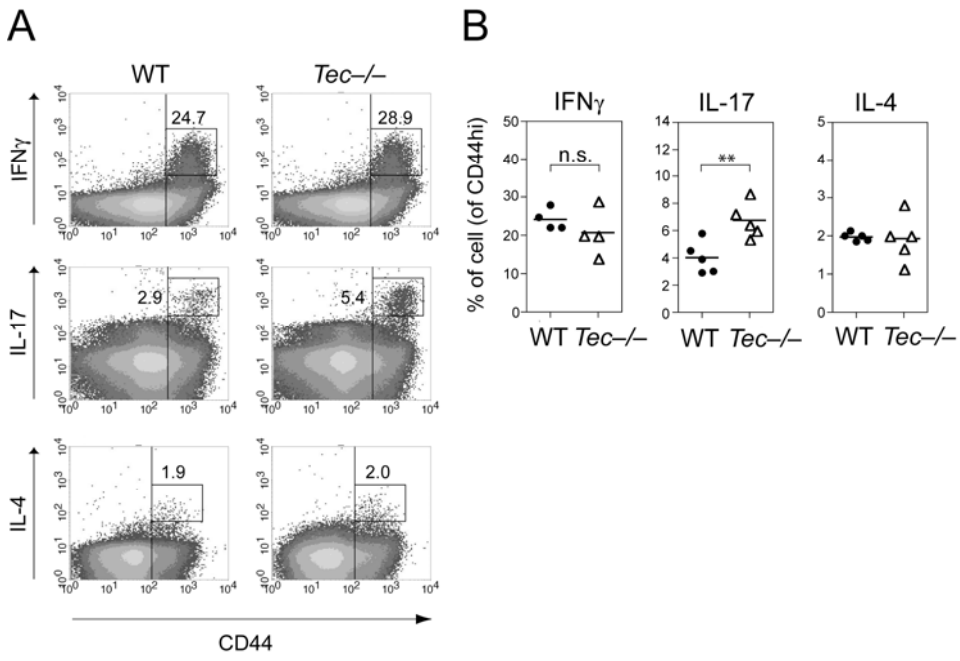

Figure 7. An IL-17-producing CD44^{hi} CD4^{+} T cells subset is increased in *Tec–/–* mice. (A) Dotplots show CD44 and intracellular IFNγ, IL-17 or IL-4 expression in *ex vivo* PMA/ionomycin stimulated purified WT and *Tec–/–* CD4^{+} T cells. (B) Diagram show the summary of all experiments performed as described in (A). The percentage of IFNγ, IL-17 and IL-4 expressing CD4^{+} T cells within the CD44^{hi} population is indicated. Each symbol represents one *ex vivo* PMA/ionomycin stimulation experiment (i.e. one mouse).

Discussion

In this study we investigated in detail the role of Tec in CD4+ T cells. We observed that Tec has no major role during T cell development and activation of naïve CD4^{+} T cells. Although Tec has been previously implicated in the CD28 co-stimulatory pathway [17,18], Tec is not required for CD28 signaling in naïve CD4^{+} T cells. Further, the differentiation of naïve CD4^{+} T cells into Th1, Th2, Th17 and regulatory T cells was normal. However, *ex vivo* PMA/ionomycin-stimulated *Tec–/–* CD4^{+} T cells contained an increased population of IL-17-producing CD44^{hi} memory-like CD4^{+} T cells, and Tec expression was strongly up-regulated in CD44^{hi} memory-like CD4^{+} T cells upon TCR

stimulation. Furthermore, activated *Tec–/–* memory-like $CD4^+$ T cells produced higher levels of IL-17 and showed increased STAT3 phosphorylation upon IL-23 stimulation compared to WT cells. Thus, our data provide a detailed description of the function of Tec in $CD4^+$ T cells and indicate a critical role for Tec in signaling pathways that regulate a population of IL-17-expressing memory-like $CD4^+$ T cells with immediate effector function.

Effector $CD4^+$ T cells can be subdivided into at least four main subsets, namely Th1, Th2, Treg, and Th17 subsets, each having a specific function in the fight against different pathogens. We did not detect any alteration in *Tec–/–* Th1 and Th2 cells with respect to IFNγ and IL-4 production, respectively. Our observation that both Th1 and Th2 cultures of Tec-deficient T cells contain high levels of IL-17, prompted us to investigate whether in the absence of Tec, Th17 cells are expanded *in vitro*. The generation of Th17 cells with TGFβ plus IL-6 from naïve *Tec–/–* $CD4^+$ T cells was comparable to WT cells. A cytokine that can inhibit Th17 differentiation is IL-2 [46]. Since Tec-deficient $CD4^+$ T cells showed slightly reduced IL-2 levels, we tested whether this could be linked to the increase in IL-17. $CD4^+$ T cells were activated in the presence of anti-murine IL-2 antibody with or without recombinant human IL-2. Preliminary results showed that blocking IL-2 rather reduces IL-17 secretion, while the addition of IL-2 had no effect (data not shown), indicating that the IL-17-producing subsets in *Tec–/–* mice are either a subset of $CD4^+$ T cells distinct from Th17 cells or already fully differentiated Th17 cells. Furthermore, IL-21 has been shown to promote Th17 differentiation [39,47-49], although this remains controversial [50,51]. We detected IL-21 in our cultures by ELISA only when memory-like $CD4^+$ T cell population were activated, however, there was no difference in IL-21 production between WT and *Tec–/–* $CD4^+$ T cells (data not shown). Thus Th17 differentiation appears to be normal in the absence of Tec. This may explain why the incidence and clinical score of EAE is normal in *Tec–/–* mice, since the development of EAE was shown to be dependent on IL-17 [52], produced mainly by MOG-sensitized $CD4^+$ T cells [43]. Additionally, the onset of allergic asthma was similar in *Tec–/–* and WT mice (data not shown). Together, these data indicate that Tec is not essential for Th1, Th2 or Th17 differentiation pathways.

This raises then the question about the T cell population that is responsible for the increased levels of IL-17 in the absence of Tec. Activated $CD4^+$ T cells as well as Th1 and Th2 cultures (that include both naïve and memory-like subsets) but not activated or Th1/Th2 differentiated naïve $CD4^+$ T cells produced IL-17 under non-polarizing conditions. This suggested that a population within the $CD44^{hi}CD62L^-$ memory-like $CD4^+$ T cell subset was affected by the absence of Tec. Indeed, Tec expression was strongly up-regulated during the activation of memory-like $CD4^+$ T

cells, and activated *Tec–/–* memory-like $CD4^+$ T cells produced higher levels of IL-17 compared to WT cells. In recent years it became clear that the $CD44^{hi}CD62L^-$ memory-like $CD4^+$ T cell population does not contain only true antigen-specific memory cells that developed in response to an antigen exposure. Rather, this population contains in addition a variety of cell subsets, some may have acquired their memory phenotype through homeostatic proliferation, while others develop in the thymus and show immediate effector function and may play a role in the defense against certain bacterial infections. These additional cells were described as memory-phenotype T cells and some subsets of them also as innate T cells [44,45]. Interestingly, Itk-deficient mice have an increase in memory-phenotype $CD4^+$ and $CD8^+$ T cells, since Itk is essential for the development of conventional (i.e. non memory-phenotype) T cells [53-57]. The observation that *ex vivo* PMA/ionomycin-stimulated *Tec–/–* $CD44^{hi}$ $CD4^+$ T cells contained a higher percentage of an IL-17-producing population compared to WT cells further indicated that a memory-phenotype or memory-like population may be affected by the loss of Tec. The role of Tec seems to be specific for an IL-17-producing T cell subset, since IFNγ-producing $CD44^{hi}$ subsets had a similar abundance in *Tec–/–* and WT cells. Thus, our data indicate a critical role for Tec in signaling pathways that regulate the generation and function of an IL-17 producing memory-like $CD4^+$ T cell subset.

A question that we have started to address is whether the increase in the IL-17-producing memory-like $CD4^+$ T cells subset is due to a T cell intrinsic defect. Tec kinase is also expressed in other lineages of the hematopoietic system [2,58]. Therefore, it is conceivable that loss of Tec could alter e.g. the pattern of cytokines secreted by these cells, which could then lead to the generation/expansion of IL-17-producing memory-like $CD4^+$ T cells. To test whether *Tec–/–* APCs induce a different T cell response compared to WT APCs, WT $CD4^+$ T cells were activated with soluble anti-CD3 in the presence of T cell-depleted APCs isolated from either WT or *Tec–/–* mice. Under these conditions, WT $CD4^+$ T cells produced similar levels of IL-17 independent whether they were stimulated with *ex vivo* isolated non-activated or activated (PGN or LPS) WT or *Tec–/–* APCs (data not shown). Taken together, these data suggest that the increase in IL-17-producing memory-like T cell population in *Tec–/–* mice is due to T cell-intrinsic alterations.

A potential mechanism by which Tec regulates IL-17-producing $CD4^+$ T cells is provided by our finding of increased expression of IL23-R. Cytokine-mediated modulation of IL-23R expression is a driving force for Th17 differentiation of naïve $CD4^+$ T cells [59]. We could show that Tec-deficient $CD4^+$ T cells (containing both $CD44^{lo}$ and $CD44^{hi}$ subsets) expressed increased levels of the *IL23r* gene upon TCR stimulation. This correlated with an increased pSTAT3 upon IL-23 stimu-

lation in memory-like $CD4^+$ T cells when they were co-cultured with naïve $CD4^+$ T cells. We also determined pSTAT3 levels in sorted and subsequently activated WT and *Tec–/–* $CD44^{hi}$ $CD4^+$ T cells. However, when cultured alone, WT memory-like cells showed a STAT3 phosphorylation comparable to Tec-deficient cells (data not shown), although IL-17 production was still higher in the absence of Tec. Thus, sorted $CD44^{hi}$ $CD4^+$ T cells show a different pSTAT3 pattern compared to cells that were cultured in the presence of naïve $CD44^{lo}$ $CD4^+$ T cells. This indicates that the naïve population might produce factors that influence $CD44^{hi}$ $CD4^+$ T cells. Further evidence that memory-like T cells are influenced by naïve $CD4^+$ T cells was obtained from our "mixed-culture" experiments, where the highest IL-17 production was observed when Tec-deficient naïve $CD4^+$ T cells were co-cultured with Tec-deficient memory-like T cells. In future experiments it will be important to investigate this potential crosstalk in more detail and to determine the factors involved.

Taken together, our study shows that Tec regulates the generation and/or function of an IL-17-producing memory-like $CD4^+$ T cell subset. Further studies aiming at a better characterization of the memory-like $CD4^+$ T cells to determine whether they represent "true" antigen-specific T cells or rather innate-like T cells are required to fully understand the role of Tec in these populations. Since IL-17 production is beneficial for immunity against extracellular pathogens like Candida albicans [60] or Klebsiella pneumoniae [61], Tec may be a potential target to increase host defense against these pathogens.

Materials and Methods

Mice

Animals used in this study were 6-12 weeks of age and bred and maintained in the animal facility of the Medical University of Vienna. *Tec-/-* mice were previously described [5,16] and backcrossed to C57BL/6 (N10). C57BL/6-Ly5.1 mice were obtained from the CDTA-CNRS, Orleans, France. All animal experiments were approved by the Federal Ministry for Science and Research.

Antibodies and cytokines

The following antibodies were from BD Pharmingen: bio-anti-CD3ε (145-2C11), bio-anti-CD11b (M1/70), bio-anti-CD11c (HL3), anti-CD28 (37.51), FITC-anti-CD4 (RM4-5), FITC-anti-CD62L (MEL 14), FITC-anti-IFNγ (XMG1.2), APC-anti-IL-2 (JES6-5H4), anti-IL12 (C17.8), anti-IL-4 (11B11), APC-anti-IL-4 (11B11), PE-anti-IL-17 (TC11-18H10), Alexa-647-anti-STAT3 (pY705), streptavidin PE-Cy7

and anti-CD16/CD32 (2.4G2). The following antibodies were from Caltag: bio-anti-CD3ε (500A2), TC-anti-CD4 (CT-CD4), FITC- or bio-anti-CD8 (5H10), PE-anti-CD25 (PC61.5.3), PE-anti-CD44 (IM.7.8.1), bio-anti-CD45R (B220), bio-anti-Ly-6G (RB6-8C5), bio-anti-erythroid cells (Ter119), and bio-anti-NK1.1 (PK136). The Alexa fluor 647-anti-mouse-FoxP3 and bio-anti-CD45.2 (104) were from eBioscience. Murine IL-4 (mIL-4) and mIL-12 were from Peprotech and mIL-6, mIL-23 and mTGFβ were from R&D. Human recombinant IL2 (rhIL-2) was a kind gift of W. Held, LICR, Lausanne.

Purification of $CD4^+$ T cells

Pooled cell suspensions of lymph nodes and spleens were incubated with biotinylated anti-CD8, anti-CD11b, anti-CD11c, anti-CD45R, anti-Ly-6G, anti-Ter119, and anti-NK antibodies in PBS/2 % FCS. The $CD4^+$ T cells were then purified by negative depletion using streptavidin beads (BD Pharmingen) according to the manufacture's instructions. The purity of the cells was assessed by flow cytometry and was routinely > 96 %. Where indicated, $CD4^+$ T cells were further sorted into $CD44^{hi}CD62L^-$ (memory-like) and $CD44^{lo}CD62L^+$ ("naïve") populations on a FACS Aria cytometer (BD).

Cell culture conditions

$CD4^+$ T Cells were grown in RPMI (Sigma), supplemented with 10% FCS (Gibco), antibiotics, L-Glutamine and 2-mercaptoethanol (Sigma).

T cell proliferation assay

Purified $CD4^+$ T cells (5 x 10^4 cells/well) were cultured for 72 h in 96-well flat-bottom plates (NUNC) coated with anti-CD3ε (0, 0.3, 1 or 10 µg/ml) in a total of 100 µl. Additional wells were coated with anti-CD3ε (1 µg/ml) plus anti-CD28 (3 µg/ml). Forty-four hours later, the cells were pulsed with [^{3}H]thymidine and harvested after 16 hours.

Calcium mobilization

T cells (1 x 10^6 /ml) were labelled for 30 min with Fluo3-AM (4 M) and FuraRed-AM (10 M) (Molecular Probes) and the Ca^{2+}-dependent fluorescence was assessed by FACS. Cells were stimulated with soluble bio-anti-CD3ε (1 µg/ml) followed by crosslinking with 5 µg/ml streptavidin (Sigma). Overall Ca^{2+} content was assessed by the addition of ionomycin (1µM; Sigma) at the end of each measurement. Data were analyzed using CELLQuest (Becton Dickinson) and FlowJo (TreeStar) software.

Semiquantitative RT-PCR analysis

cDNA was prepared from *in vitro* activated $CD4^+$ T cells after 6 days in culture, restimulated for 8 hours with plate-bound anti-CD3 and analyzed by semiquantitative PCR [20] via the following primers: *Hprt*-F: 5'-ATTGTGGCCCTCTGTGTGCT, *Hprt*-R: 5'-TTGCGCTCATCTTAGGCTTTG, *Il23r*-F: 5'-TTCTGCGTCCATCATTTCCA**,** *Il23r*-R: 5'-CCATTCCCGACAAAAGTCCA.

Differentiation and activation of the various $CD4^+$ T cell lineages

Purified $CD4^+$ T cells were stimulated with plate-bound anti-CD3ε (1 μg/ml) and plate-bound anti-CD28 (3 μg/ml) on 48 well plates (0.5 to 1 x 10^6 cells/well). For non-polarizing conditions, cells were activated in the presence of rhIL-2 (20 U/ml). For Th1 polarizing conditions, cells were activated in the presence of rhIL-2 (20 U/ml), IL-12 (5 ng/ml), and anti-IL-4 (3 μg/ml). For Th2 polarizing conditions, cells were activated in the presence of recombinant IL-4 (250 U/ml), anti-IFNγ (10 μg/ml) and anti-IL12 (10 μg/ml). For Th17 differentiation, sorted naïve $CD4^+$ T cells were activated in the presence of IL-6 (20 ng/ml), and TGFβ1 (1 ng/ml). Regulatory T cells (Treg) were generated from sorted naive $CD4^+$ T cells with TGFβ1 (1 ng/ml). $CD4^+$ T cell cultures were split 1:2 on day 3 after activation, and rested for additional 2 to 3 days, except for Th17 and Treg cells that were cultured only for 4 days. Unless otherwise indicated, cells were purified at the end of the culture period over a lymphoprep gradient, and 0,5 x 10^5 cells were added on 96 well plates coated with or without anti-CD3ε (1 μg/ml). Supernatant for cytokine quantification was collected 12 h later. In some experiments, sorted naïve and memory-like $CD4^+$ T cell subsets from WT and Tec-deficient mice were co-cultures at a 14:1 ratio in different combinations as described in Figure 5A. The "mixed-cultures" were then activated and stimulated under non-polarizing conditions as described above.

Cytokine measurement

IL-2, IL-4 and IFNγ cytokine levels were determined by ELISA (BD). For IL-17 ELISA, capture (anti-IL17, clone TC11-18H10) and detection (bio-anti-IL17, clone TC11-8H4.1) antibodies were from BD. IL-17 standard was from eBioscience.

Intracellular stainings

For the detection of intracellular cytokines, $CD4^+$ T cells that have been cultured under various conditions or that were freshly isolated and purified were stimulated at 10^6 cells/ml for 4 h with PMA (50 ng/ml) and Ionomycin (500 ng/ml) (Sigma) in presence of GolgiSTOP (BD). Cells were first incubated with TC-anti-CD4, then fixed with 2 % paraformaldehyde (PFA) and permeabilized

with Perm/wash solution (BD Pharmingen). Cells were further incubated with FITC-anti-IFNγ, PE-anti-IL-17, and APC-anti-IL-4 or Alexa-647-anti-Foxp3 or APC-anti-IL-2 and washed with 2% FCS in PBS. Samples were acquired on a FACSCalibur (BD) and data were analyzed with FlowJo software (Treestar) and CellQuest (BD).

Intracellular detection of STAT3 phosphorylation

Ly5.2$^+$ WT or *Tec–/–* CD44^{hi}CD62L$^-$ (memory-like) CD4$^+$ T cells, and Ly5.1$^+$ WT CD44^{lo}CD62L$^+$ (naïve) CD4$^+$ T cells were isolated by sorting. Then, naive Ly5.1$^+$ WT cells were co-cultured with either WT or *Tec–/–* memory-like cells at a ratio that exists in WT or *Tec–/–* mice (approx. 14:1). The mixed cell populations were stimulated (under the same conditions as total CD4$^+$ T cells), rested, starved in RPMI/1% FCS and reactivated at 10^7 cells/ml in RPMI/1% FCS with IL-23 (10 ng/ml) or IL-6 (10 ng/ml) for 15 minutes. The reaction was stopped by addition of 16 % PFA, yielding a final concentration of 2 % PFA. Cells were left for 20 minutes in PFA and were then permeabilized for 20 minutes with methanol at a final concentration of 80 %. Cells were washed twice with PBS/2 % FCS and stained with FITC-anti-CD4, biot-anti-Ly5.2 and Alexa-647- anti-phospho(Y705)STAT3. Cells were washed, incubated with PE-Cy7-streptavidin. Data were acquired on a FACSCalibur (BD) and evaluated using FloJo (Treestar).

Western blotting

T cells were pelleted and lyzed in Carin lysis buffer (20 mM Tris-HCl pH 8.0, 138 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 % NP40, 10 % Glycerol, 2 mM NA-Vanadate) supplemented with complete protease inhibitors (Roche). Proteins were then separated on 10 % SDS-polyacrylamide gels and blotted onto PVDF membranes. The anti-phosphotyrosine antibody 4G10 was from Upstate. Anti-PLC□1 and anti-P-STAT3 (Y701) antibodies were from Cell Signalling. The rabbit anti-C-terminal Tec was a kind gift of H. Mano, Jichi Medical School, Japan. Anti-STAT3 was from Santa Cruz. Secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

For STAT3 immunoblots, cells were purified after 6 days in culture over a lymphoprep gradient, starved for 1 h in medium containing 1% FCS, and stimulated at 10^7 cells/ml for various time points with IL-23 (10 ng/ml).

Induction of EAE

Mice were injected subcutaneously with 100 □l of an emulsion consisting of heat-killed *M. tuberculosis*-supplemented (strain H37Ra) CFA (DIFCO) and 50 □g MOG peptide (amino acids 35–55). Mice received 200 ng Pertussis toxin (Sigma) intraperitoneally on the day of immunization and 2

d later. EAE was assessed daily, and clinical scores were assigned according to the following criteria: 0, unaffected; 1, flaccid tail; 2, impaired righting reflex and/or gait; 3, partial hindlimb paralysis; 4, total hind limb paralysis; 5, total hindlimb paralysis with partial forelimb paralysis; 6, death. For histological analysis, mice were perfused on day 14.

Statistical analysis

All data are expressed as the mean ± SD. Statistical analysis was performed by using a Student`s t test. The P-values were defined as following: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

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Authorship and Conflict of Interest Statements

N.B., W.E. designed research

N.B., B.V., J.R., A.C. A.S., U.S. performed experiments

N.B., A.C., J.B., R.B., M.M.E. H.L., A.W., W.E. designed, performed and/or analyzed *in vivo* disease models

N.B., W.E. wrote the paper

References

1. Samelson LE. Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. Annu Rev Immunol. 2002;20:371-394.

2. Schmidt U, Boucheron N, Unger B, Ellmeier W. The role of Tec family kinases in myeloid cells. Int Arch Allergy Immunol. 2004;134:65-78.

3. Berg LJ, Finkelstein LD, Lucas JA, Schwartzberg PL. Tec family kinases in T lymphocyte development and function. Annu Rev Immunol. 2005;23:549-600.

4. Schwartzberg PL, Finkelstein LD, Readinger JA. TEC-family kinases: regulators of T-helper-cell differentiation. Nat Rev Immunol. 2005;5:284-295.

5. Liao XC, Littman DR. Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. Immunity. 1995;3:757-769.

6. Liu KQ, Bunnell SC, Gurniak CB, Berg LJ. T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. J Exp Med. 1998;187:1721-1727.

7. Schaeffer EM, Broussard C, Debnath J, Anderson S, McVicar DW, Schwartzberg PL. Tec family kinases modulate thresholds for thymocyte development and selection. J Exp Med. 2000;192:987-1000.

8. Fowell DJ, Shinkai K, Liao XC, et al. Impaired NFATc translocation and failure of Th2 development in Itk-deficient CD4+ T cells. Immunity. 1999;11:399-409.

9. Schaeffer EM, Yap GS, Lewis CM, et al. Mutation of Tec family kinases alters T helper cell differentiation. Nat Immunol. 2001;2:1183-1188.

10. Miller AT, Wilcox HM, Lai Z, Berg LJ. Signaling through Itk promotes T helper 2 differentiation via negative regulation of T-bet. Immunity. 2004;21:67-80.

11. Woods ML, Shimizu Y. Signaling networks regulating beta1 integrin-mediated adhesion of T lymphocytes to extracellular matrix. J Leukoc Biol. 2001;69:874-880.

12. Dombroski D, Houghtling RA, Labno CM, et al. Kinase-independent functions for Itk in TCR-induced regulation of Vav and the actin cytoskeleton. J Immunol. 2005;174:1385-1392.

13. Grasis JA, Browne CD, Tsoukas CD. Inducible T cell tyrosine kinase regulates actin-dependent cytoskeletal events induced by the T cell antigen receptor. J Immunol. 2003;170:3971-3976.

14. Labno CM, Lewis CM, You D, et al. Itk functions to control actin polymerization at the immune synapse through localized activation of Cdc42 and WASP. Curr Biol. 2003;13:1619-1624.

15. Schaeffer EM, Debnath J, Yap G, et al. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science. 1999;284:638-641.

16. Ellmeier W, Jung S, Sunshine MJ, et al. Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. J Exp Med. 2000;192:1611-1624.

17. Yang WC, Ghiotto M, Barbarat B, Olive D. The role of Tec protein-tyrosine kinase in T cell signaling. J Biol Chem. 1999;274:607-617.

18. Yang WC, Olive D. Tec kinase is involved in transcriptional regulation of IL-2 and IL-4 in the CD28 pathway. Eur J Immunol. 1999;29:1842-1849.

19. Tomlinson MG, Kane LP, Su J, Kadlecek TA, Mollenauer MN, Weiss A. Expression and function of Tec, Itk, and Btk in lymphocytes: evidence for a unique role for Tec. Mol Cell Biol. 2004;24:2455-2466.

20. Schebesta A, McManus S, Salvagiotto G, Delogu A, Busslinger GA, Busslinger M. Transcription factor Pax5 activates the chromatin of key genes involved in B cell signaling, adhesion, migration, and immune function. Immunity. 2007;27:49-63.

21. August A, Gibson S, Kawakami Y, Kawakami T, Mills GB, Dupont B. CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. Proc Natl Acad Sci U S A. 1994;91:9347-9351.

22. Liao XC, Fournier S, Killeen N, Weiss A, Allison JP, Littman DR. Itk negatively regulates induction of T cell proliferation by CD28 costimulation. J Exp Med. 1997;186:221-228.

23. Mustelin T, Tasken K. Positive and negative regulation of T-cell activation through kinases and phosphatases. Biochem J. 2003;371:15-27.

24. Irvin BJ, Williams BL, Nilson AE, Maynor HO, Abraham RT. Pleiotropic contributions of phospholipase C-gamma1 (PLC-gamma1) to T-cell antigen receptor-mediated signaling: reconstitution studies of a PLC-gamma1-deficient Jurkat T-cell line. Mol Cell Biol. 2000;20:9149-9161.

25. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005;6:1123-1132.

26. Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol. 2005;6:1133-1141.

27. Ivanov, II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006;126:1121-1133.

28. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity. 2008;28:454-467.

29. McGeachy MJ, Cua DJ. Th17 cell differentiation: the long and winding road. Immunity. 2008;28:445-453.

30. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 Family Cytokines and the Expanding Diversity of Effector T Cell Lineages. Annu Rev Immunol. 2007;25:821-852.

31. Cho ML, Kang JW, Moon YM, et al. STAT3 and NF-kappaB signal pathway is required for IL-23-mediated IL-17 production in spontaneous arthritis animal model IL-1 receptor antagonist-deficient mice. J Immunol. 2006;176:5652-5661.

32. Chen Z, Laurence A, Kanno Y, et al. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. Proc Natl Acad Sci U S A. 2006;103:8137-8142.

33. Kimura A, Naka T, Kishimoto T. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. Proc Natl Acad Sci U S A. 2007.

34. Mathur AN, Chang HC, Zisoulis DG, et al. Stat3 and Stat4 direct development of IL-17-secreting Th cells. J Immunol. 2007;178:4901-4907.

35. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006;24:179-189.

36. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441:235-238.

37. Mangan PR, Harrington LE, O'Quinn DB, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature. 2006;441:231-234.

38. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity. 2004;21:467-476.

39. Nurieva R, Yang XO, Martinez G, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature. 2007;448:480-483.

40. Liang SC, Tan XY, Luxenberg DP, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med. 2006;203:2271-2279.

41. Zheng Y, Danilenko DM, Valdez P, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature. 2007;445:648-651.

42. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med. 2005;201:233-240.

43. Komiyama Y, Nakae S, Matsuki T, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol. 2006;177:566-573.

44. Surh CD, Boyman O, Purton JF, Sprent J. Homeostasis of memory T cells. Immunol Rev. 2006;211:154-163.

45. Berg LJ. Signalling through TEC kinases regulates conventional versus innate CD8(+) T-cell development. Nat Rev Immunol. 2007;7:479-485.

46. Laurence A, Tato CM, Davidson TS, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity. 2007;26:371-381.

47. Korn T, Bettelli E, Gao W, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007;448:484-487.

48. Wei L, Laurence A, Elias KM, O'Shea JJ. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. J Biol Chem. 2007;282:34605-34610.

49. Yang L, Anderson DE, Baecher-Allan C, et al. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. Nature. 2008.

50. Coquet JM, Chakravarti S, Smyth MJ, Godfrey DI. Cutting Edge: IL-21 Is Not Essential for Th17 Differentiation or Experimental Autoimmune Encephalomyelitis. J Immunol. 2008;180:7097-7101.

51. Suto A, Kashiwakuma D, Kagami SI, et al. Development and characterization of IL-21-producing CD4+ T cells. J Exp Med. 2008.

52. Hofstetter HH, Ibrahim SM, Koczan D, et al. Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. Cell Immunol. 2005;237:123-130.

53. Atherly LO, Lucas JA, Felices M, Yin CC, Reiner SL, Berg LJ. The Tec family tyrosine kinases Itk and Rlk regulate the development of conventional CD8+ T cells. Immunity. 2006;25:79-91.

54. Broussard C, Fleischacker C, Fleischecker C, et al. Altered development of CD8+ T cell lineages in mice deficient for the Tec kinases Itk and Rlk. Immunity. 2006;25:93-104.

55. Dubois S, Waldmann TA, Müller JR. ITK and IL-15 support two distinct subsets of CD8+ T cells. Proc Natl Acad Sci USA. 2006;103:12075-12080.

56. Hu J, Sahu N, Walsh E, August A. Memory phenotype CD8+ T cells with innate function selectively develop in the absence of active Itk. Eur J Immunol. 2007;37:2892-2899.

57. Hu J, August A. Naive and Innate Memory Phenotype CD4+ T Cells Have Different Requirements for Active Itk for Their Development. J Immunol. 2008;180:6544-6552.

58. Mano H. Tec family of protein-tyrosine kinases: an overview of their structure and function. Cytokine Growth Factor Rev. 1999;10:267-280.

59. Zhou L, Ivanov, II, Spolski R, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol. 2007;8:967-974.

60. Huang W, Na L, Fidel PL, Schwarzenberger P. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. J Infect Dis. 2004;190:624-631.

61. Ye P, Rodriguez FH, Kanaly S, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med. 2001;194:519-527.

3.4 The role of Tec family kinases in inflammatory processes

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The Role of Tec Family Kinases in Inflammatory Processes

Martin Melcher, Julia Raberger†, Uwe Schmidt†, Bernd Unger† and Wilfried Ellmeier*

Institute of Immunology, Medical University of Vienna, Lazarettgasse 19, A-1090 Vienna, Austria

Abstract: A variety of different cell lineages of the hematopoietic system are activated during inflammatory processes. These cells not only contribute to the beneficial outcome of an immune response, but can also cause pathology such as autoimmunity as a consequence of extended or uncontrolled reactions. Therefore, an understanding of the basic mechanisms that lead to immune cell activation and the identification of key molecular players will also lead to strategies towards therapeutic manipulation of extended immune reactions. Members of the Tec kinase family (Bmx, Btk, Itk, Rlk and Tec) constitute an important class of non-receptor protein tyrosine kinases that are primarily expressed in the hematopoietic system. They are activated upon a variety of signals and are important participants of major signal transduction pathways in immunological processes. Hence, deficiencies in Tec family kinases cause several immunological defects, both in man and mice. Since Tec family kinases have been shown to function as modulators of immune cell activation, they may provide attractive drug targets for the manipulation of the immune response. In this review, we summarize recent data from studies about the activities of Tec family kinases in inflammatory cells and their role in *in vivo* models of infection, inflammation and autoimmune diseases.

Keywords: Tec family kinases, inflammation, mouse models, immunity, Th1/Th2.

INTRODUCTION

The inflammatory response is a continuous sequence of a variety of different processes. After detection of infection or tissue damage, the inflammatory process begins with changes in tissue homeostasis and blood flow, and the migration of immune cells in waves comprised of different cell types to the site of pathogen exposure or tissue trauma. In a normal situation, the recruited inflammatory cells remove pathogens or cellular debris. Subsequently, the immune cells themselves are removed leading to the cessation of the inflammatory response. However, any alterations in the balance between the activities of the inflammatory cells can cause pathology, leading to diseases such as chronic inflammation, asthma and arthritis [1].

At a cellular level, various cells of the immune system participate in the inflammatory response. The local release of mediators such as cytokines and chemokines at the site of infection and injury leads to the recruitment and activation of neutrophils and subsequently macrophages, which themselves are then activated by the presence of cytokines or bacterial components such as LPS. This leads to the initiation of various signal transduction cascades within these cells and the subsequent activation of their microbicidal activities. The activation of non-receptor protein tyrosine kinases (PTK) is one of the first steps in the signal transduction cascades that lead to activation of immune cells upon binding of a ligand to its receptor. One class of non-receptor PTK that is activated by a variety of signals present at the inflammatory site are the Tec family kinases, which form the second largest family of non-receptor PTK in the hematopoietic system. Several recent reviews summarize the current knowledge about the structure, activation pathways and function of Tec family kinases in cells of the innate and adaptive immune system [2-4]. In this review we focus on the *in vivo* role of immune cells deficient for members of the Tec family kinase and summarize recent studies using mouse models for inflammatory diseases and autoimmunity.

A BRIEF OVERVIEW ABOUT TEC FAMILY KINASES AND THEIR FUNCTIONS IN SIGNAL TRANSDUCTION CASCADES

The name giving member of this kinase family, Tec (tyrosine kinase expressed in hepatocellular carcinoma), was identified in 1990 [5]. In addition to Tec, four additional mammalian members of the Tec kinase family have been described: Bmx (Bone marrow kinase on the X chromosome; also named Etk) [6-8], Btk (Bruton's tyrosine kinase) [9-11], Itk (IL-2-inducible T cell kinase; also known as Emt) [6, 12, 13], and Rlk (Resting lymphocyte kinase; also designated Txk) [14, 15]. The expression of Tec family kinases is restricted primarily to the hematopoietic system (for a review see [4]), although expression of Bmx and Tec has been detected in endothelial cells [7] and liver [5], respectively. Tec family kinases came into the focus of immunological interest in 1993 when it was shown that a broad range of mutations in one member of this family, Btk, are the cause of X-linked agammaglobulinemia (XLA) in humans [10, 11]. XLA is an immunodeficiency characterized by a severe reduction of serum immunoglobulin (Ig) levels due to defects in B lymphocyte development and function. A similar although less severe syndrome in the mouse, X-linked immunodeficiency (*xid*), is caused by a point mutation in the *btk* gene [9]. The most characteristic feature of Tec family kinases (with the exception of Rlk; see below) is a pleckstrin homology (PH)

*Address correspondence to this author at the Institute of Immunology, Medical University of Vienna, Lazarettgasse 19, A-1090 Vienna, Austria. Tel: +43-1-40160-65003; Fax: +43-1-40160-965006;
Email: wilfried.ellmeier@meduniwien.ac.at

† *These authors contributed equally*

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domain located at the N-terminus of the molecule (see Fig. (**1**)). The PH domain is followed by a short so-called Tec homology (TH) domain, and by the Src homology (SH) domains SH3 and SH2, known to interact with proline-rich sequences or to bind to sequences containing phosphorylated tyrosines, respectively [16, 17]. In contrast to Src family kinases, Tec family kinases do not have a C-terminal negative regulatory tyrosine residue, indicating different regulatory modes. PH domains, found in a number of intracellular signaling molecules, are able to bind phospholipids or proteins [18]. TH domains, consisting of a Btk homology (BH) motif and of one or two proline-rich (PR) regions, have been implicated in the auto-regulation of Tec kinases [19]. The TH and SH3 domains of Itk are able to interact intramolecularly, thus preventing the interaction of these domains with their prospective ligands. Furthermore, binding of the PR region of Tec family kinases to Src family kinases has been demonstrated [20]. Rlk represents an atypical Tec family kinase member since it lacks the PH domain and the BH motif. Instead, Rlk contains an N-terminal palmitoylated cystein rich sequence that anchors Rlk to the membrane [21].

Tec family kinases have been most extensively analyzed in the lymphoid lineages. A large number of biochemical, functional and genetic studies, in particular focusing on Itk and Btk, have shown that the activation of Tec family kinases upon antigen receptor stimulation requires several key regulatory steps (for detailed recent reviews on the activation of Tec family kinases and their role in T and B cells, see [2, 3]). These regulatory steps involve regulation by subcellular localization, phosphorylation and colocalization with membrane associated adapter proteins and intra- and intermolecular interactions. In a first step of the activation of Tec family kinases, the molecule has to be localized to the plasma membrane, a process that is mediated by the interaction of the PH domain with phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) generated by PI3-Kinase (PI3-K) activity. The interaction of the PH domain with PIP_3 targets Tec family kinases to specific membrane microdomains (lipid rafts or glycolipid-enriched membrane microdomains, GEM) [22-25]. Rlk does not contain a PH domain, however Rlk can be anchored to the membrane via palmitoylation of a N-terminal cysteine-string motif [21]. This leads to a constitutive localization of Rlk to lipid rafts [26]. Upon localization of Tec family kinases to the membrane, a tyrosine residue within the activation loop of the kinase domain is phosphorylated by Src family kinases, which subsequently, upon autophosphorylation of a tyrosine residue within the SH3 domain, results in the full activation of Tec kinases (see [2, 3] and references therein). In addition to translocation and phosphorylation, the activity of Tec family kinases is also regulated by their interaction with adaptor molecules such as SLP-76 and SLP-65 [27] and LAT (via Grb2 [19, 22]), leading to recruitment and integration of Tec family kinases into BCR- or TCR-initiated signaling pathways. As mentioned

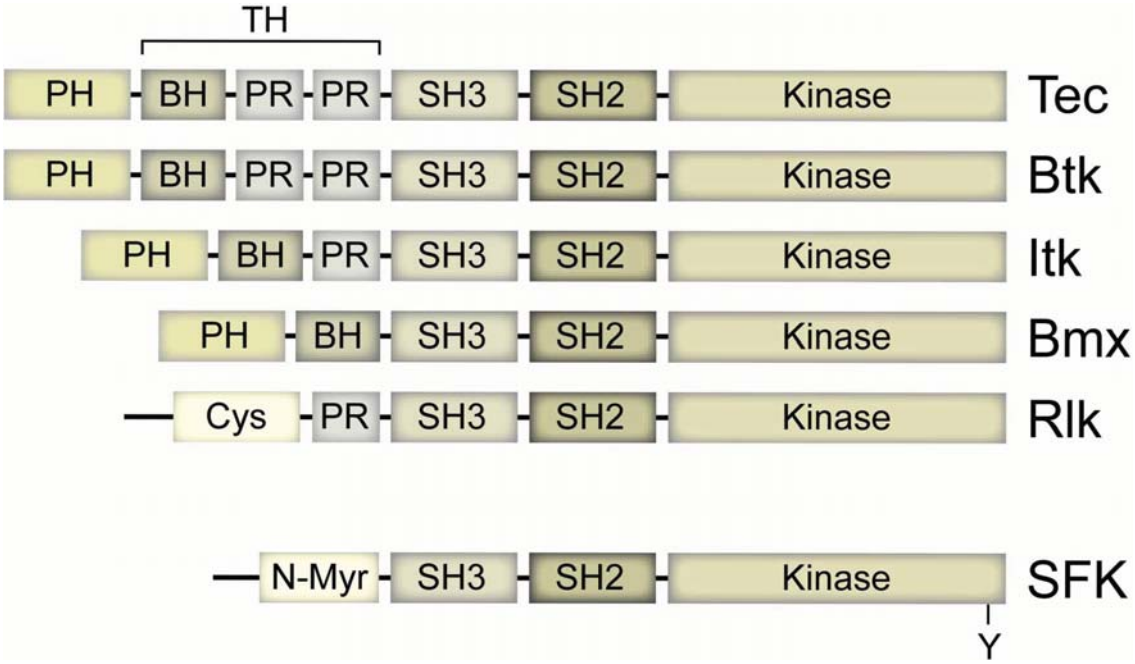

Fig. (1). Schematic map of Tec family kinases in comparison to Src family kinases.
The hallmark of Tec family kinases is the PH domain at the N-terminus, shown to mediate protein-phospholipid and protein-protein interactions. The TH domain, formed by the BH motif and the PR region(s), is involved in the autoregulation of Tec family kinases. The SH3 domain binds to proline-rich sequences, and the SH2 domain to phosphotyrosine residues. Although Rlk lacks the PH domain and the BH motif characteristic for Tec family kinases, the high sequence homology between the SH domains of Rlk and of other Tec family kinases classifies Rlk as member of the Tec kinase family. In contrast to Src kinases (SFK), Tec family kinases do not have a negative regulatory tyrosine residue at the C-terminus. See text for further details. Abbreviations: BH, Btk homology motif; Cys, cysteine-rich sequence; N-Myr, N-terminal myristilation signal; PH, pleckstrin homology domain; PR, proline-rich region; SFK, Src family kinases; SH, src homology domain; TH, Tec homology domain.

above, the TH domain (i.e. the PR within the TH domain) and the SH3 domain of Itk are able to interact intramolecularly, thus preventing the binding of these domains to potential ligands [19] and adding another potential layer of regulation. Similar observations have also been made for Btk and Tec, although they form preferentially homodimers via reciprocal TH/SH3 domain-mediated intermolecular interactions [2].

Biochemical studies of either *Btk–/–* or *xid* mice, human B cells derived from XLA patients or Btk-deficient chicken DT40 B cells indicate that Btk is required for full tyrosine phosphorylation and activation of phospholipase Cγ2. As a consequence, Btk-deficient B cells show a reduced intracellular Ca^{2+} mobilization [28, 29]. Similar observations have been described in Itk-deficient and Itk/Rlk double-deficient T cells [30-32], and in mast cells lacking Btk [33]. Beside their role in Ca^{2+} mobilization, Tec family kinases have been implicated in the regulation of apoptosis, the reorganization of the actin cytoskeleton, integrin activation during the formation of the immunological synapse, and cell adhesion and migration processes (for detailed reviews see [34, 35]). Furthermore, Tec family kinases have been linked to the regulation of gene expression, not only indirectly by causing a reduction of the activity of Ca^{2+}-sensitive transcription factors of the NFAT family, but also due to their interaction with transcription factors such as Stat3 [36], Stat5 [37], BAP-135/TFII-I [38] and bright [39]. Btk [39], Itk [40] and Rlk [21] can localize to the nucleus, which may connect them via interaction with transcriptional regulators directly to the regulation of transcriptional activation. In line with these findings, it has even been demonstrated that Rlk can act as a Th-1-specific transcription factor that participates in the regulation of IFNγ expression [41]. Finally, Itk has been implicated in regulating the activity of the Th1 key regulatory factor T-bet. T-bet can interact with the Th2 master regulator GATA-3 and thereby interferes with GATA-3 activity leading to the repression of Th2 commitment. In order to interact with GATA-3, T-bet has to be tyrosine phosphorylated, and Itk mediates this phospohorylation. This indicates that Itk-dependent phosphorylation can regulate the interaction of two opposing transcription factors [42].

NEUTROPHIL FUNCTIONS IN THE ABSENCE OF TEC FAMILY KINASES

Tec family kinases are not only critical components of antigen receptor signaling pathways in lymphocytes, but are also activated in cells of the myeloid lineage by a variety of receptor signaling pathways such as integrin activation, Fc receptors, G-protein-coupled receptors, Toll-like receptors (TLR), death receptors and cytokine receptors [4]. Among the cells of the myeloid cell lineages, neutrophils are key cellular components of the inflammatory process. They are among the first cells being recruited to the site of inflammation. One of the main tasks of recruited neutrophils is the removal of pathogens from the site of inflammation (for a review see [43]). They can be activated by bacterial components (like LPS or formylated peptides), which induce a number of neutrophil functions such as migration, phagocytosis and the production of microbicidal substances like reactive oxygen metabolites. Activated neutrophils that do not encounter any pathogens release their microbicidal effector substances after a certain time [44]. This creates a toxic environment destroying pathogens, but can also lead to the destruction of extracellular matrix molecules and tissue damage. Furthermore, chemotactic signals generated by neutrophils attract monocytes and dendritic cells and influence macrophage differentiation towards a pro- or anti-inflammatory state (see [43] and references therein).

So far only very few studies addressed the role of Tec family kinases in neutrophils. Human neutrophils express Bmx, Btk and Tec [45]. Stimulation with the chemotactic factor fMLP induces membrane translocation and phosphorylation of all three kinases [45]. Crosslinking of the receptor CD16b (FcγIIIB) on human neutrophils leads to the phosphorylation and translocation of Tec to the plasma membrane [46]. Btk might also influence neutrophil development at least under certain circumstances. XLA patients show an increased incidence of neutropenia in association with infection. This indicates that Btk, although not normally required for neutrophil production, might be required during inflammation and infection [47]. Further indications for a role of Btk in neutrophils came from experiments using Carragenan-induced edema as a model of neutrophil-dependent acute inflammation [48]. In this inflammation model, *xid* mice show reduced footpad edema and neutrophils from *xid* mice show an impaired induction of reactive oxygen intermediates (ROI) and nitric oxide (NO) compared to neutrophils from control mice [49]. Using the Btk inhibitor LFM-A13 to block Btk activity [50], it has been shown *in vitro* that neutrophils have reduced tyrosine phosphorylation patterns upon fMLP stimulation in the presence of the inhibitor [51]. LFM-A13 inhibits the adhesion and chemotaxis of neutrophils and the production of superoxide anion [51]. LFM-A13 has been reported also to inhibit degranulation of neutrophils upon CD16b crosslinking [46]. However, since it has recently been shown that LFM-A13 is also a potent inhibitor of Jak2 [52] and of Tec [46], some additional studies using complementary approaches (such as RNAi) to inactivate Btk and/or Tec in human neutrophils should be performed to confirm the proposed role of Btk and/or Tec in neutrophils.

THE ROLE OF TEC FAMILY KINASES IN MONOCYTES/MACROPHAGES AND DENDRITIC CELLS

Macrophages are large phagocytic mononuclear cells and an important cell type in the inflammatory response. They constitute an extremely heterogenous population distributed over the whole body and are derived from the same bone marrow precursors as dendritic cells and osteoclasts. Monocytes, the precursor of tissue macrophages, reside in the bloodstream. A subset of monocytes, upon migration into tissues, differentiates into resident tissue macrophages [53], which together with other stromal cells are capable of initiating the early stages of an inflammatory response [54, 55]. Another subset of monocytes is recruited upon inflammation and matures into inflammatory macrophages [56]. Macrophages are involved in virtually all inflammatory processes from the detection of pathogens up to the resolution of inflammation and repair of tissue damage. They have a wide array of surface receptors to detect pathogens, examples include complement and Fc receptors and TLRs. Apart from

Table 1. Tec Family Kinase-Deficient Mice Tested in *In Vivo* Models for Infections, Inflammation and Autoimmunity

Disease model / infection	Genotype	Results/phenotype and references
Experimental autoimmune encephalomyelitis (EAE)	*xid*	• Slower induction of EAE and clinically less severe [49, 73]
Carragenan-induced edema	*xid*	• Reduced footpad edema due to impaired neutrophil function [49]
Dextran-sodium sulphate induced colitis	*xid*	• No weight loss upon induction of colitis [49]
Collagen-induced arthritis (CIA)	*xid*	• resistant to the induction of CIA on a DBA1 background [72]
Allergic asthma	*Itk–/–*	• Attenuated symptoms of allergic asthma, reduced eosinophila infiltration, reduced mucus production [81, 107] • Impaired $CD4^+$ T cell function and cytokine production [81, 107] • Reduced contractile response [111] • Impaired airway mast cell degranulation [81]
Allergic asthma	wildtype	• Itk inhibitor BMS-509744 shows dose dependent reduction of lung inflammation [113]
Allergic asthma	*Btk–/–*	• One study reports exaggerated airway inflammation [77], while another one reports no differences [81]
Early and late phase passive cutaneous anaphylactic reaction	Btk–/– *xid*	• Reduced early and late phase reaction [33, 81]
Infection with *Setaria digitata*	*xid*	• Delayed clearance of filarial infection due to poor nitrite oxide production in macrophages [68, 119]
Infection with *Nippostrongylus brasiliensis*	*xid*	• Increased IgE response [79]
Infection with *Schistosoma mansoni*	*xid*	• On a Balb/c background higher susceptibility to infection and higher amounts of IgE and IgG1 antibodies [80]
Infection with *Trypanosoma cruzi*	*xid*	• On a Balb/c background poor B cell responses, however mice can control parasitemia [120]
Infection with *Leishmania major*	*xid*	• On a Balb/c background resistance to *L. major* [121]
Infection with *Toxoplasma gondii*	*Itk–/–* *Itk–/–Rlk–/–*	• Graded defects on lethality upon infection, *Itk–/–Rlk–/–* mice are more severely affected than *Itk–/–* mice [31]
Infection with *Schistosoma mansoni eggs*	*Itk–/–* *Itk–/–Rlk–/–*	• *Itk–/–* mice have decrease Th2 response, however *Itk–/–Rlk–/–* mice display normal Th2 response [100]
Infection with *Leishmania major*	*Itk–/–*	• On a Balb/c background development of a Th1 instead of a Th2 response and therefore mice are capable of clearing the intramacrophage parasite (unlike wildtype control mice) [99]
Infection with lymphocytic choriomeningitis virus (LCMV), vaccinia virus (VV), and vesicular stomatitis virus (VSV)	*Itk–/–* *Itk–/–Rlk–/–*	• Normal elimination of LCMV in *Itk–/–*, *Itk–/–Rlk–/–* and control mice [101, 102] • VV is cleared with delayed kinetics in *Itk–/–* mice [101] • Antibody responses to VSV infection are similar in *Itk–/–* and control mice [101] • *Ex vivo* CTL responses to LCMV are reduced in *Itk–/–* mice [101] • Reduced numbers of LCMV-specific $CD8^+$ T cells in *Itk–/–* and *Itk–/–Rlk–/–* mice [102] • Impaired accumulation of $CD8^+$ T cells in response to LCMV or VV infection in *Itk–/–* and *Itk–/–Rlk–/–* mice [102]

Table listing the various infection, inflammation and autoimmune disease models applied to Tec family kinase-deficient mice together with the observed phenotypes and the citations of the studies performing the experiments.

clearing infectious microorganisms, inflammatory macrophages can also initiate the destruction of extracellular matrix and the death of stromal, parenchymal and other immune cells. However, they are also involved in the secretion and stabilization of new matrix molecules and wound healing [57].

Three members of the Tec kinase family, Btk, Tec and Bmx, have been shown to be expressed in macrophages [58-60]. Functional analysis of macrophages and biochemical studies of signaling pathways activated in the above mentioned processes have shed light on the role of Tec family kinases in macrophages during inflammation. Fc receptor- and complement receptor-dependent phagocytosis and che-

motaxis are impaired in monocytes derived from XLA patients [61]. Tec family kinases have also been implicated in TLR signaling (for a review see [62]). Btk interacts with the TIR region of TLR2, 4, 8 and 9 as well as the adaptor molecules MyD88 and MyD88-adaptor-like protein (Mal) [63]. Btk also binds to IRAK-1 but not TRAF6, both important molecules of the TLR signalosome [63-65]. Furthermore Btk is activated by the TLR4-ligand LPS in the human promonocytic cell line THP-1 and dominant-negative Btk abrogates LPS activation of NF-κB in U973 cells and the murine monocytic cell line RAW264.7 [63]. Btk is also activated in mouse peritoneal macrophages during the zymosan- or Staphylococcus aureus-induced release of arachidonate, the precursor of leukotrienes and prostaglandins, which are early mediators of inflammation. In addition, arachidonate release can be reduced by LFM-A13, further suggesting a role for Btk in this process [66]. Tec family kinases are also implicated in TLR-induced signaling pathways and the subsequent induction of effector functions like cytokine production. *Xid* macrophages show impaired p65 phosphorylation and transactivation upon LPS stimulation, while IkBα-degradation is normal [67]. Furthermore, *xid* macrophages exhibit an impaired secretion of the proinflammatory cytokines TNFα and IL-1β after stimulation with LPS [68]. In humans, monocytes from XLA patients have been shown to be impaired in the production of TNFα and IL-1β upon stimulation of the TLR4 or TLR2 [69], although another study reports that Btk is not essential for early LPS signaling [70]. Overexpression of Btk in normal human monocytes leads to the stabilization of TNFαmRNA and therefore to an increase in TNFα production [58, 69]. Interestingly, incubation of XLA monocytes with M-CSF leads to an increase in the expression of Tec and restores their ability to produce TNFα upon LPS stimulation [58]. This suggests a compensatory role for Tec, similar to the situation observed in murine B cells [71], which may also explain why XLA patients show normal innate immune responses [58].

Functional studies of macrophages from *xid* mice have shown that Btk is required for efficient effector function. *Xid* macrophages produce less of the effector molecule nitric oxide after induction with various stimuli. Macrophages from *xid* mice are also incapable of producing efficient bursts of ROI [49]. The impaired production of effector molecules or of pro-inflammatory cytokines might explain the delayed clearance of microfilarial infections *in vivo* in *xid* mice [68]. It has been shown that *xid* mice are less susceptible to autoimmune and inflammatory diseases [72, 73]. These defects have mostly been attributed to B cell defects, but could also indicate a defect in macrophage function. Recently, *xid* mice have been tested in several models for induced inflammatory disease [49]. The development of experimental autoimmune encephalomyelitis (EAE) shows slower induction and is clinically less severe in *xid* mice as compared to wildtype controls, even though *xid* mice exhibit a Th1 bias due to impaired macrophage antigen presenting function [49, 74]. A similar reduction of disease severity in *xid* mice was also observed in dextran sodium sulphate-induced colitis and Carrageenan-induced acute edema, two inflammation models that are independent of the adaptive immune system. Taken together, the decreased severity of several *in vivo* inflammation models in *xid* mice can be attributed to impaired myeloid cell function due to the absence of functional Btk.

Btk is not only involved in the activation of TLR induced inflammation but is also critical for the dampening of an inflammatory response. Mal is phosphorylated by Btk [75] and this phosphorylation is crucial for its interaction with SOCS-1. This interaction results in the polyubiquitination of Mal and its degradation by the 26S proteosome [76]. In splenocytes derived from *xid* mice, Mal is not degraded upon TLR4 triggering, which leads to increased Mal-dependent NFκB transactivation and potentiates TLR signaling and the pro-inflammatory response [76].

Another example of Btk as a negative regulator was demonstrated in bone marrow-derived dendritic cells (BMDC). *Btk–/–* BMDCs exhibit a more mature phenotype and have an enhanced *in vitro* and *in vivo* T cell stimulatory capacity [77]. *Btk–/–* BMDCs produce reduced levels of the immunosuppressive cytokine IL-10 compared to their wildtype counterparts and show impaired phosphorylation of Stat3 after LPS stimulation. Since IL-10 has been shown to induce Stat3-phosphorylation [78], these studies suggest that IL-10 production and subsequent Stat3 activation is regulated by Btk-dependent signaling pathways that negatively regulate the maturation and T cell stimulatory capacity of BMDCs [77]. Using an allergic airway inflammation model, it has been reported that *Btk–/–* mice have an increased IgE response, exaggerated airway inflammation and a general enhancement of Th1 and Th2 responses [77]. Furthermore, adoptive transfer of antigen loaded *Btk–/–* BMDCs leads to increased IgE levels in recipients as compared to the transfer of wildtype BMDCs [77], providing a potential explanation for the observation of high IgE responses in *xid* mice upon infection [79, 80]. However, another study did not observe increased airway inflammation in *Btk–/–* mice [81]. Possible explanations for these differences could be different immunization protocols and/or different genetic backgrounds of the mice used for the studies.

MAST CELLS

Mast cells are well-accepted key players in pathological processes such as allergic asthma or systemic anaphylaxis. These disorders are characterized by uncontrolled antigen dependent activation of mast cells via cross-linking of the high-affinity IgE receptor (FcεRI). Activated mast cells can release a tremendous amount of mediators that if out of control can lead to severe damage or even destruction of the respective tissues. A large body of evidence demonstrates that beside the FcεRI signalosome, mast cell activation can occur via alternative pathways and that mast cells also influence innate and adaptive immune responses [82]. Mast cells are also required for the full development of autoimmune diseases in models such as MOG-induced EAE or arthritis [83-86] and have been implicated in the development of inflammatory bowel disease [87]. Thus inhibition of mast cell effector functions may represent one attractive avenue to target these pathological processes.

Mast cells express four out of five Tec family members (Btk, Itk, Rlk and Tec) and Btk, Itk and Tec are activated upon FcεRI ligation [88, 89]; (Schmidt and Ellmeier, unpublished data). Studies with Btk-deficient mast cells (either

from *Btk–/–* or *xid* mice) demonstrated that loss of Btk activity results in impaired histamine secretion, a mild impairment of total leukotriene synthesis and in a more severely impaired cytokine production/secretion *in vitro* [33, 90, 91]. *In vivo*, impaired mast cell functions of *Btk–/–* mice were demonstrated by passive cutaneous anaphylactic (PCA) reactions using both an early and late PCA model. Early extravasation of Evan´s blue dye is significantly reduced in a dose dependent manner and Btk-deficient mice fail to induce skin edema [33]. A role for Itk during mast cell activation has been reported recently. Using an OVA-induced acute phase plasma extravasation model, it was shown that *Itk–/–* mice exhibit a more pronounced reduction in the response as compared to Btk-deficient and wildtype control mice [81]. In line with this finding, *Itk–/–* airway mast cells also show a more severely decreased degranulation response in comparison to Btk-deficient and wildtype airway mast cells [81]. The contribution of Tec to mast cell biology is currently under investigation (U.Schmidt & W.Ellmeier, manuscript in preparation). To our best knowledge, there exist no reports describing a function for Rlk in mast cells.

T CELLS AND TEC FAMILY KINASES IN ADAPTIVE IMMUNE RESPONSES AND DURING INFLAMMATION

$CD4^+$ T cells have been traditionally classified into Th1 and Th2 cell lineages. Th1/Th2 helper cell differentiation is regulated by the cytokine environment and lineage specific transcription factors. The transcription factor T-bet is a master regulator of Th1 cell differentiation, while GATA3 is important for Th2 cell development. Th1 cells produce IFNγ and regulate cell-mediated immunity, whereas Th2 cells produce IL-4, IL-5 and IL-13 and mediate the humoral arm of the immune response and also allergic responses (for reviews see [92, 93]). Recent data indicate the existence of a third subset of T helper cells that mainly produces IL-17 [94, 95]. It has been proposed that these so-called Th17 cells have crucial roles in regulating tissue inflammation and the occurrence of disease in several animal models of autoimmunity (for review see [96, 97]).

Tec family kinases are critical components of TCR-mediated signaling pathways and crucial regulators of Th1 and Th2 cell differentiation pathways (for a review see [98]). Infection of *Itk–/–Rlk–/–* and *Itk–/–* mice with Toxoplasma gondii display graded resistance against the pathogen with *Itk–/–Rlk–/–* mice showing the most reduced survival rate followed by *Itk–/–* mice compared to wildtype controls, indicating *in vivo* defects in T cell function [31]. It has been shown that Itk-deficient T cells are not able to produce efficient amounts of IL-4 even if they are cultivated under Th2 skewing conditions [99]. *In vivo*, *Itk–/–* mice on a Balb/c background do not, unlike wildtype Balb/c mice, develop a Th2 response upon infection with *Leishmania major*. Instead, they develop a Th1 response and can clear the infection of the intramacrophagal parasite [99]. In a different infection model, *Itk–/–* mice also show a decreased Th2 response when challenged with *Schistosoma mansoni* eggs. In contrast, *Rlk–/–Itk–/–* mice are able to mount a protective Th2 response upon challenge with *Schistosoma mansoni* eggs [100]. The mechanism of the restoration of the Th2 response in *Rlk–/–Itk–/–* mice is not clear, however several possibilities have been recently discussed [98].

A few studies have also tested whether *Rlk–/–Itk–/–* and *Itk–/–* mice are able to mount a protective response against viral infection [101, 102]. *Itk–/–* mice were infected either with lymphocytic choriomeningitis virus (LCMV), vaccinia virus (VV) or vesicular stomatitis virus (VSV) [101]. LCMV is eliminated by day 8 in both *Itk–/–* and control mice, while VV is cleared with delayed kinetics in *Itk–/–* mice. Antibody responses to VSV infection are similar in *Itk–/–* and control mice. However, *ex vivo* primary cytotoxic T-lymphocyte responses against LCMV, VV or VSV are reduced after infection of *Itk–/–* mice, showing a reduction of CTL responses in the absence of Itk in contrast to antiviral B cell responses. Antiviral responses to LCMV have also been tested in *Itk–/–Rlk–/–* mice. Similar to *Itk–/–* mice, double-deficient mice are also able to clear the virus, even though the accumulation of antigen-specific $CD8^+$ T cells is reduced in both *Itk–/–* and *Itk–/–Rlk–/–* mice compared to wildtype controls [102].

Since Itk-deficient mice have an impaired Th2 response, it has been tested whether Itk could be a potential target in the treatment of allergic asthma. Allergic asthma is characterized by an increase of Th2 cells and Th2 type cytokines in the lung, increased mucus secretion and inflammation of the airways [103]. Transfer of antigen-specific Th2 cells or introduction of IL-4 and IL-13 alone can lead to airway hyperresponsiveness along with all the symptoms of allergic asthma [104, 105]. Blocking the activity of these cytokines, as also shown for IL-4 in humans, reduces the development of disease [104, 106]. In an animal model for allergic asthma, *Itk–/–* mice exhibit attenuated immunological symptoms. *Itk–/–* mice display reduced eosinophil infiltration into the lung, show a reduced thickening of the epithelial cell layer lining the bronchioles, and also have reduced mucus secretion after induction of allergic asthma [81, 107]. Mice lacking Itk also show reduced antigen-specific recruitment of T cells into the lung and a diminished secretion of IL-4, IL-5 and IL-13 by Itk-deficient T cells [107]. The reason for the reduced T cell infiltration is not clear, however multiple factors could contribute to the defects. Since chemokines are required for the recruitment of T cells into the lung in this model system [108], it has been speculated that the reduced T cell numbers might be due to altered chemokine/chemokine-receptor signaling in *Itk–/–* T cells leading to impaired migration [109, 110]. Furthermore, adhesion defects of *Itk–/–* T cells could contribute to the reduced numbers of T cells in the lung [25]. More recent observations show that isolated trachea from Itk-deficient mice have a reduced contractile response to stimulation with cholinergic agonists and depolarizing agents [111]. Since Itk is not expressed in trachea and smooth muscle, it has been suggested that the reduced contractile response in Itk-deficient mice could be due to the different local trachea cytokine milieu. One of the involved cytokines could be IL-13, which is known to affect contractile responses of smooth muscle cells [112], since reduced IL-13 mRNA levels are observed in the lungs of Itk-deficient mice [111]. In addition, the disease can be induced in *Itk–/–* mice by adoptively transferring antigen-specific wildtype $CD4^+$ T cells, thus confirming the major role of T cells in the development of allergic asthma [111].

Finally, the Itk Inhibitor BMS-509744 has already been evaluated successfully in a murine allergic asthma model. This inhibitor is able to reduce lung inflammation in a dose dependent manner leading to a reduction of both total cell and eosinophil infiltration into the lung [113], thus supporting the concept that Tec family kinases are suitable targets for therapeutic intervention in inflammatory diseases.

SMALL MOLECULE INHIBITORS FOR KINASE-DEPENDENT AND KINASE-INDEPENDENT ACTIVITIES OF TEC FAMILY KINASES

As mentioned above, inhibitors of Tec family kinases have been already identified. These inhibitors are directed against the kinase domain and can successfully impair its activity. However, kinase-independent functions for Btk and Itk have been reported as well. Kinase-dead Btk mutants can partially rescue the B cell developmental defects of *Btk–/–* mice [114] and the tumor suppressor function of Btk in the absence of SLP-65 is also independent of its kinase activity [115]. Kinase-dead Btk is also capable of stimulating Calcium influx in B cells upon BCR stimulation [116]. Furthermore, kinase-dead Itk can rescue TCR-induced Vav localization and actin polarization in Itk-deficient T cells [117], and a mutant form of Itk that lacks the kinase domain can partially rescue antigen receptor signalling in *Btk–/–* DT-40 cells [118]. Taken together, these data indicate kinase-independent functions and suggest that Tec family kinases may also act as adaptor and scaffolding molecules. Therefore, optimal strategies using small molecule inhibitors to inactivate Tec family kinases should not only be directed against the catalytic domain but should also take into account the potential adaptor/scaffolding function of Tec family kinases.

SUMMARY

A large number of studies performed during the last decade have convincingly indicated that Tec family kinases have a crucial role in cells of both the innate and adaptive branch of the immune system and therefore also in inflammatory processes. Tec family kinases are activated by a variety of extracellular signals and are involved in cellular functions essential to inflammation, such as differentiation, activation, adhesion, death and migration. Lymphoid and myeloid cell lineages express multiple members of Tec family kinases and genetic experiments have shown both unique and compensatory functions among Tec family kinases. In the near future combinatorial Tec family kinase knockout mice can be expected to provide a clear picture of the functions of these kinases in the hematopoietic system, in immunological processes in general and during inflammation in particular. Finally, some studies to determine whether and how Tec family kinase deficiency affects the outcome of experimentally-induced models for inflammation and autoimmune diseases have already been performed and indicate that Tec family kinases are indeed promising targets for the treatment of inflammatory diseases. Thus, a better and broader understanding of the *in vivo* response of various Tec family kinase-deficient mice in several disease models will help to select particular members of Tec family kinases as candidates for rational drug design.

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REFERENCES

[1] Henson, P. M. *Nat. Immunol.*, **2005**, *6*, 1179.
[2] Berg, L. J.; Finkelstein, L. D.; Lucas, J. A.; Schwartzberg, P. L. *Annu. Rev. Immunol.*, **2005**, *23*, 549.
[3] Lindvall, J. M.; Blomberg, K. E.; Valiaho, J.; Vargas, L.; Heinonen, J. E.; Berglof, A.; Mohamed, A. J.; Nore, B. F.; Vihinen, M.; Smith, C. I. *Immunol. Rev.*, **2005**, 203, 200.
[4] Schmidt, U.; Boucheron, N.; Unger, B.; Ellmeier, W. *Int. Arch. Allergy Immunol.*, **2004**, *134*, 65.
[5] Mano, H.; Ishikawa, F.; Nishida, J.; Hirai, H.; Takaku, F. *Oncogene*, **1990**, *5*, 1781.
[6] Yamada, N.; Kawakami, Y.; Kimura, H.; Fukamachi, H.; Baier, G.; Altman, A.; Kato, T.; Inagaki, Y.; Kawakami, T. *Biochem. Biophys. Res. Commun.*, **1993**, *192*, 231.
[7] Tamagnone, L.; Lahtinen, I.; Mustonen, T.; Virtaneva, K.; Francis, F.; Muscatelli, F.; Alitalo, R.; Smith, C. I.; Larsson, C.; Alitalo, K. *Oncogene,* **1994**, *9*, 3683.
[8] Robinson, D.; He, F.; Pretlow, T.; Kung, H. J. *Proc. Natl. Acad. Sci. USA,* **1996**, *93*, 5958.
[9] Rawlings, D. J.; Saffran, D. C.; Tsukada, S.; Largaespada, D. A.; Grimaldi, J. C.; Cohen, L.; Mohr, R. N.; Bazan, J. F.; Howard, M.; Copeland, N. G.; Jenkins, N.A.; Witte, O.N. *Science*, **1993**, *261*, 358.
[10] Tsukada, S.; Saffran, D. C.; Rawlings, D. J.; Parolini, O.; Allen, R. C.; Klisak, I.; Sparkes, R. S.; Kubagawa, H.; Mohandas, T.; Quan, S.; Belmont, J.W.; Cooper, M.D.; Conley, M.E.; Witte, O.N. *Cell*, **1993**, *72*, 279.
[11] Vetrie, D.; Vorechovsky, I.; Sideras, P.; Holland, J.; Davies, A.; Flinter, F.; Hammarstrom, L.; Kinnon, C.; Levinsky, R.; Bobrow, M.; Smith, E.; Bentley, D.R. *Nature*, **1993**, *361*, 226.
[12] Siliciano, J. D.; Morrow, T. A.; Desiderio, S. V. *Proc. Natl. Acad. Sci.* U S A, **1992**, 89, 11194.
[13] Heyeck, S. D.; Berg, L. J. *Proc. Natl. Acad. Sci.* U S A, **1993**, *90*, 669.
[14] Haire, R. N.; Ohta, Y.; Lewis, J. E.; Fu, S. M.; Kroisel, P.; Litman, G. W. *Hum. Mol. Genet.*, **1994**, *3*, 897.
[15] Hu, Q.; Davidson, D.; Schwartzberg, P. L.; Macchiarini, F.; Lenardo, M. J.; Bluestone, J. A.; Matis, L. A. *J. Biol. Chem.*, **1995**, *270*, 1928.
[16] Schlessinger, J. *Curr. Opin. Genet. Dev.*, **1994**, *4*, 25.
[17] Mayer, B. J.; Gupta, R. Curr. Top. Microbiol. Immunol., **1998**, *228*, 1.
[18] Smith, C. I.; Islam, T. C.; Mattsson, P. T.; Mohamed, A. J.; Nore, B. F.; Vihinen, M. *Bioessays*, **2001**, *23*, 436.
[19] Andreotti, A. H.; Bunnell, S. C.; Feng, S.; Berg, L. J.; Schreiber, S. L. *Nature*, **1997**, *385*, 93.
[20] Cheng, G.; Ye, Z. S.; Baltimore, D. *Proc. Natl. Acad. Sci.* U S A, **1994**, *91*, 8152.
[21] Debnath, J.; Chamorro, M.; Czar, M. J.; Schaeffer, E. M.; Lenardo, M. J.; Varmus, H. E.; Schwartzberg, P. L. *Mol. Cell. Biol.*, **1999**, *19*, 1498.
[22] Bunnell, S. C.; Diehn, M.; Yaffe, M. B.; Findell, P. R.; Cantley, L. C.; Berg, L. J. *J. Biol. Chem.*, **2000**, *275*, 2219.
[23] Guo, B.; Kato, R. M.; Garcia-Lloret, M.; Wahl, M. I.; Rawlings, D. J. *Immunity*, **2000**, *13*, 243.
[24] Shan, X.; Czar, M. J.; Bunnell, S. C.; Liu, P.; Liu, Y.; Schwartzberg, P. L.; Wange, R. L. *Mol. Cell. Biol.*, **2000**, *20*, 6945.
[25] Woods, M. L.; Kivens, W. J.; Adelsman, M. A.; Qiu, Y.; August, A.; Shimizu, Y. *EMBO J.*, **2001**, *20*, 1232.

[26] Chamorro, M.; Czar, M. J.; Debnath, J.; Cheng, G.; Lenardo, M. J.; Varmus, H. E.; Schwartzberg, P. L. *BMC Immunol.*, **2001**, *2*, 3.
[27] Su, Y. W.; Zhang, Y.; Schweikert, J.; Koretzky, G. A.; Reth, M.; Wienands, J. *Eur. J. Immunol.*, **1999**, *29*, 3702.
[28] Takata, M.; Kurosaki, T. *J. Exp. Med.*, **1996**, *184*, 31.
[29] Fluckiger, A. C.; Li, Z.; Kato, R. M.; Wahl, M. I.; Ochs, H. D.; Longnecker, R.; Kinet, J. P.; Witte, O. N.; Scharenberg, A. M.; Rawlings, D. J. *EMBO J.*, **1998**, *17*, 1973.
[30] Liu, K. Q.; Bunnell, S. C.; Gurniak, C. B.; Berg, L. J. *J. Exp. Med.*, **1998**, *187*, 1721.
[31] Schaeffer, E. M.; Debnath, J.; Yap, G.; McVicar, D.; Liao, X. C.; Littman, D. R.; Sher, A.; Varmus, H. E.; Lenardo, M. J.; Schwartzberg, P. L. *Science*, **1999**, *284*, 638.
[32] Donnadieu, E.; Lang, V.; Bismuth, G.; Ellmeier, W.; Acuto, O.; Michel, F.; Trautmann, A. *J. Immunol.*, **2001**, *166*, 5540.
[33] Hata, D.; Kawakami, Y.; Inagaki, N.; Lantz, C. S.; Kitamura, T.; Khan, W. N.; Maeda-Yamamoto, M.; Miura, T.; Han, W.; Hartman, S. E.; Yao, L.; Nagai, H.; Goldfeld, A. E.; Alt, F. W.; Galli, S. J.; Witte, O. N.; Kawakami, T. *J. Exp. Med.*, **1998**, *187*, 1235.
[34] Finkelstein, L. D.; Schwartzberg, P. L. *Trends Cell Biol.*, **2004**, *14*, 443.
[35] Takesono, A.; Finkelstein, L. D.; Schwartzberg, P. L. *J. Cell Sci.*, **2002**, *115*, 3039.
[36] Tsai, Y. T.; Su, Y. H.; Fang, S. S.; Huang, T. N.; Qiu, Y.; Jou, Y. S.; Shih, H. M.; Kung, H. J.; Chen, R. H. *Mol. Cell. Biol.*, **2000**, *20*, 2043.
[37] Mahajan, S.; Vassilev, A.; Sun, N.; Ozer, Z.; Mao, C.; Uckun, F. M. *J. Biol. Chem.*, **2001**, *276*, 31216.
[38] Yang, W.; Desiderio, S. *Proc. Natl. Acad. Sci.* U S A, **1997**, *94*, 604.
[39] Webb, C. F.; Yamashita, Y.; Ayers, N.; Evetts, S.; Paulin, Y.; Conley, M. E.; Smith, E. A. *J. Immunol.*, **2000**, *165*, 6956.
[40] Perez-Villar, J. J.; O'Day, K.; Hewgill, D. H.; Nadler, S. G.; Kanner, S. B. *Int. Immunol.*, **2001**, *13*, 1265.
[41] Takeba, Y.; Nagafuchi, H.; Takeno, M.; Kashiwakura, J.; Suzuki, N. *J. Immunol.*, **2002**, *168*, 2365.
[42] Hwang, E. S.; Szabo, S. J.; Schwartzberg, P. L.; Glimcher, L. H. *Science*, **2005**, *307*, 430.
[43] Nathan, C. *Nat. Rev. Immunol.*, **2006**, *6*, 173.
[44] Nathan, C. F. *J. Clin. Invest.*, 1987, *80*, 1550.
[45] Lachance, G.; Levasseur, S.; Naccache, P. H. *J. Biol. Chem.*, **2002**, *277*, 21537.
[46] Fernandes, M. J.; Lachance, G.; Pare, G.; Rollet-Labelle, E.; Naccache, P. H. *J. Leukoc. Biol.*, **2005**, *78*, 524.
[47] Farrar, J. E.; Rohrer, J.; Conley, M. E. *Clin. Immunol. Immunopathol.*, **1996**, *81*, 271.
[48] Szabo, C.; Lim, L. H.; Cuzzocrea, S.; Getting, S. J.; Zingarelli, B.; Flower, R. J.; Salzman, A. L.; Perretti, M. *J. Exp. Med.*, **1997**, *186*, 1041.
[49] Mangla, A.; Khare, A.; Vineeth, V.; Panday, N. N.; Mukhopadhyay, A.; Ravindran, B.; Bal, V.; George, A.; Rath, S. *Blood*, **2004**, *104*, 1191.
[50] Mahajan, S.; Ghosh, S.; Sudbeck, E. A.; Zheng, Y.; Downs, S.; Hupke, M.; Uckun, F. M. *J. Biol. Chem.*, **1999**, *274*, 9587.
[51] Gilbert, C.; Levasseur, S.; Desaulniers, P.; Dusseault, A. A.; Thibault, N.; Bourgoin, S. G.; Naccache, P. H. *J. Immunol.*, **2003**, *170*, 5235.
[52] van den Akker, E.; van Dijk, T. B.; Schmidt, U.; Felida, L.; Beug, H.; Lowenberg, B.; von Lindern, M. *Biol. Chem.*, **2004**, *385*, 409.
[53] Gordon, S. *Nat. Rev. Immunol.*, **2003**, *3*, 23.
[54] Cailhier, J. F.; Partolina, M.; Vuthoori, S.; Wu, S.; Ko, K.; Watson, S.; Savill, J.; Hughes, J.; Lang, R. A. *J. Immunol.*, **2005**, *174*, 2336.
[55] Cailhier, J. F.; Sawatzky, D. A.; Kipari, T.; Houlberg, K.; Walbaum, D.; Watson, S.; Lang, R. A.; Clay, S.; Kluth, D.; Savill, J.; Hughes, *J. Am. J. Respir. Crit. Care Med.*, **2006**, *173*, 540.
[56] Geissmann, F.; Jung, S.; Littman, D. R. *Immunity*, **2003**, *19*, 71.
[57] Duffield, J. S. *Clin. Sci.*. (Lond), **2003**, *104*, 27.
[58] Horwood, N. J.; Mahon, T.; McDaid, J. P.; Campbell, J.; Mano, H.; Brennan, F. M.; Webster, D.; Foxwell, B. M. *J. Exp. Med.*, **2003**, *197*, 1603.
[59] Kaukonen, J.; Lahtinen, I.; Laine, S.; Alitalo, K.; Palotie, A. Br. J. Haematol., **1996**, *94*, 455.
[60] Weil, D.; Power, M. A.; Smith, S. I.; Li, C. L. *Blood*, **1997**, *90*, 4332.
[61] Amoras, A. L.; Kanegane, H.; Miyawaki, T.; Vilela, M. M. *J. Investig. Allergol. Clin. Immunol.*, **2003**, *13*, 181.
[62] Jefferies, C. A.; O'Neill, L. A. *Immunol. Lett.*, **2004**, *92*, 15.
[63] Jefferies, C. A.; Doyle, S.; Brunner, C.; Dunne, A.; Brint, E.; Wietek, C.; Walch, E.; Wirth, T.; O'Neill, L. A. *J. Biol. Chem.*, **2003**, *278*, 26258.
[64] Fitzgerald, K. A.; Palsson-McDermott, E. M.; Bowie, A. G.; Jefferies, C. A.; Mansell, A. S.; Brady, G.; Brint, E.; Dunne, A.; Gray, P.; Harte, M. T.; McMurray, D.; Smith, D. E.; Sims, J. E.; Bird, T. A.; O'Neill, L. A. *Nature*, **2001**, *413*, 78.
[65] Medzhitov, R. *Nat. Rev. Immunol.*, **2001**, *1*, 135.
[66] Olsson, S.; Sundler, R. *J. Inflamm.* (Lond), **2006**, *3*, 8.
[67] Doyle, S. L.; Jefferies, C. A.; O'Neill, L. A. *J. Biol. Chem.*, **2005**, *280*, 23496.
[68] Mukhopadhyay, S.; Mohanty, M.; Mangla, A.; George, A.; Bal, V.; Rath, S.; Ravindran, B. *J. Immunol.*, **2002**, *168*, 2914.
[69] Horwood, N. J.; Page, T. H.; McDaid, J. P.; Palmer, C. D.; Campbell, J.; Mahon, T.; Brennan, F. M.; Webster, D.; Foxwell, B. M. *J. Immunol.*, **2006**, *176*, 3635.
[70] Perez de Diego, R.; Lopez-Granados, E.; Pozo, M.; Rodriguez, C.; Sabina, P.; Ferreira, A.; Fontan, G.; Garcia-Rodriguez, M. C.; Alemany, S. *J. Allergy Clin. Immunol.*, **2006**, *117*, 1462.
[71] Ellmeier, W.; Jung, S.; Sunshine, M. J.; Hatam, F.; Xu, Y.; Baltimore, D.; Mano, H.; Littman, D. R. *J. Exp. Med.*, **2000**, *192*, 1611.
[72] Jansson, L.; Holmdahl, R. *Clin. Exp. Immunol.*, **1993**, *94*, 459.
[73] Svensson, L.; Abdul-Majid, K. B.; Bauer, J.; Lassmann, H.; Harris, R. A.; Holmdahl, R. *Eur. J. Immunol.*, **2002**, *32*, 1939.
[74] Mukhopadhyay, S.; Sahoo, P. K.; George, A.; Bal, V.; Rath, S.; Ravindran, B. *J. Immunol.*, **1999**, *163*, 875.
[75] Gray, P.; Dunne, A.; Brikos, C.; Jefferies, C. A.; Doyle, S. L.; O'Neill, L. A. *J. Biol. Chem.*, **2006**, *281*, 10489.
[76] Mansell, A.; Smith, R.; Doyle, S. L.; Gray, P.; Fenner, J. E.; Crack, P. J.; Nicholson, S. E.; Hilton, D. J.; O'Neill, L. A.; Hertzog, P. J. *Nat. Immunol.*, **2006**, *7*, 148.
[77] Kawakami, Y.; Inagaki, N.; Salek-Ardakani, S.; Kitaura, J.; Tanaka, H.; Nagao, K.; Xiao, W.; Nagai, H.; Croft, M.; Kawakami, T. *Proc. Natl. Acad. Sci.* U S A, **2006**, *103*, 153.
[78] Finbloom, D. S.; Winestock, K. D. *J. Immunol.*, **1995**, *155*, 1079.
[79] Lebrun, P.; Sidman, C. L.; Spiegelberg, H. L. *J. Immunol.*, ***1988***, *141*, 249.
[80] Gaubert, S.; Viana da Costa, A.; Maurage, C. A.; Lima, E. C.; Fontaine, J.; Lafitte, S.; Minoprio, P.; Capron, A.; Grzych, J. M. *Parasite Immunol.*, **1999**, *21*, 89.
[81] Forssell, J.; Sideras, P.; Eriksson, C.; Malm-Erjefalt, M.; Rydell-Tormanen, K.; Ericsson, P. O.; Erjefalt, J. S. *Am. J. Respir. Cell Mol. Biol.*, **2005**, *32*, 511.
[82] Galli, S. J.; Nakae, S.; Tsai, M. *Nat. Immunol.*, **2005**, *6*, 135.
[83] Steinman, L. *Nat. Immunol.*, **2001**, *2*, 762.
[84] Robbie-Ryan, M.; Tanzola, M. B.; Secor, V. H.; Brown, M. A. *J. Immunol.*, **2003**, *170*, 1630.
[85] Lee, D. M.; Friend, D. S.; Gurish, M. F.; Benoist, C.; Mathis, D.; Brenner, M. B. *Science*, **2002**, *297*, 1689.
[86] Secor, V. H.; Secor, W. E.; Gutekunst, C. A.; Brown, M. A. *J. Exp. Med.*, **2000**, *191*, 813.
[87] Rijnierse, A.; Koster, A. S.; Nijkamp, F. P.; Kraneveld, A. D. *J. Immunol.*, **2006**, *176*, 4375.
[88] Kawakami, Y.; Yao, L.; Miura, T.; Tsukada, S.; Witte, O. N.; Kawakami, T. *Mol. Cell. Biol.*, **1994**, *14*, 5108.
[89] Kawakami, Y.; Yao, L.; Tashiro, M.; Gibson, S.; Mills, G. B.; Kawakami, T. *J. Immunol.*, **1995**, *155*, 3556.
[90] Setoguchi, R.; Kinashi, T.; Sagara, H.; Hirosawa, K.; Takatsu, K. *Immunol. Lett.*, **1998**, *64*, 109.
[91] Iwaki, S.; Tkaczyk, C.; Satterthwaite, A. B.; Halcomb, K.; Beaven, M. A.; Metcalfe, D. D.; Gilfillan, A. M. *J. Biol. Chem.*, **2005**, *280*, 40261.
[92] Glimcher, L. H.; Murphy, K. M. *Genes Dev.*, **2000**, *14*, 1693.
[93] Murphy, K. M.; Reiner, S. L. *Nat. Rev. Immunol.*, **2002**, *2*, 933.
[94] Harrington, L. E.; Hatton, R. D.; Mangan, P. R.; Turner, H.; Murphy, T. L.; Murphy, K. M.; Weaver, C. T. *Nat. Immunol.*, **2005**, *6*, 1123.
[95] Park, H.; Li, Z.; Yang, X. O.; Chang, S. H.; Nurieva, R.; Wang, Y. H.; Wang, Y.; Hood, L.; Zhu, Z.; Tian, Q.; Dong, C. *Nat. Immunol.*, **2005**, *6*, 1133.
[96] Dong, C. *Nat. Rev. Immunol.*, **2006**, *6*, 329.
[97] Weaver, C. T.; Harrington, L. E.; Mangan, P. R.; Gavrieli, M.; Murphy, K. M. *Immunity*, **2006**, *24*, 677.
[98] Schwartzberg, P. L.; Finkelstein, L. D.; Readinger, J. A. *Nat. Rev. Immunol.*, **2005**, *5*, 284.

[99] Fowell, D. J.; Shinkai, K.; Liao, X. C.; Beebe, A. M.; Coffman, R. L.; Littman, D. R.; Locksley, R. M. *Immunity*, **1999**, *11*, 399.
[100] Schaeffer, E. M.; Yap, G. S.; Lewis, C. M.; Czar, M. J.; McVicar, D. W.; Cheever, A. W.; Sher, A.; Schwartzberg, P. L. *Nat. Immunol.*, **2001**, *2*, 1183.
[101] Bachmann, M. F.; Littman, D. R.; Liao, X. C. *J. Virol.*, **1997**, *71*, 7253.
[102] Atherly, L. O.; Brehm, M. A.; Welsh, R. M.; Berg, L. J. *J. Immunol.*, **2006**, *176*, 1571.
[103] Cohn, L.; Elias, J. A.; Chupp, G. L. *Annu. Rev. Immunol.*, **2004**, *22*, 789.
[104] Grunig, G.; Warnock, M.; Wakil, A. E.; Venkayya, R.; Brombacher, F.; Rennick, D. M.; Sheppard, D.; Mohrs, M.; Donaldson, D. D.; Locksley, R. M.; Corry, D. B. *Science*, **1998**, *282*, 2261.
[105] Zheng, T.; Zhu, Z.; Wang, Z.; Homer, R. J.; Ma, B.; Riese, R. J., Jr.; Chapman, H. A., Jr.; Shapiro, S. D.; Elias, J. A. *J. Clin. Invest.*, **2000**, *106*, 1081.
[106] Borish, L. C.; Nelson, H. S.; Lanz, M. J.; Claussen, L.; Whitmore, J. B.; Agosti, J. M.; Garrison, L. *Am. J. Respir. Crit. Care Med.*, **1999**, *160*, 1816.
[107] Mueller, C.; August, A. *J. Immunol.*, **2003**, *170*, 5056.
[108] Gonzalo, J. A.; Lloyd, C. M.; Kremer, L.; Finger, E.; Martinez, A. C.; Siegelman, M. H.; Cybulsky, M.; Gutierrez-Ramos, J. C. *J. Clin. Invest.*, **1996**, *98*, 2332.
[109] Fischer, A. M.; Mercer, J. C.; Iyer, A.; Ragin, M. J.; August, A. *J. Biol. Chem.*, **2004**, *279*, 29816.
[110] Takesono, A.; Horai, R.; Mandai, M.; Dombroski, D.; Schwartzberg, P. L. *Curr. Biol.*, **2004**, *14*, 917.
[111] Ferrara, T. J.; Mueller, C.; Sahu, N.; Ben-Jebria, A.; August, A. *J. Allergy Clin. Immunol.*, **2006**, *117*, 780.
[112] Tliba, O.; Deshpande, D.; Chen, H.; Van Besien, C.; Kannan, M.; Panettieri, R. A., Jr.; Amrani, Y. *Br. J. Pharmacol.*, **2003**, *140*, 1159.
[113] Lin, T. A.; McIntyre, K. W.; Das, J.; Liu, C.; O'Day, K. D.; Penhallow, B.; Hung, C. Y.; Whitney, G. S.; Shuster, D. J.; Yang, X.; Townsend, R.; Postelnek, J.; Spergel, S. H.; Lin, J.; Moquin, R. V.; Furch, J. A.; Kamath, A. V.; Zhang, H.; Marathe, P. H.; Perez-Villar, J. J.; Doweyko, A.; Killar, L.; Dodd, J. H.; Barrish, J. C.; Wityak, J.; Kanner, S. B. *Biochemistry*, **2004**, *43*, 11056.
[114] Middendorp, S.; Dingjan, G. M.; Maas, A.; Dahlenborg, K.; Hendriks, R. W. *J. Immunol.*, **2003**, *171*, 5988.
[115] Middendorp, S.; Zijlstra, A. J.; Kersseboom, R.; Dingjan, G. M.; Jumaa, H.; Hendriks, R. W. *Blood*, **2005**, *105*, 259.
[116] Saito, K.; Tolias, K. F.; Saci, A.; Koon, H. B.; Humphries, L. A.; Scharenberg, A.; Rawlings, D. J.; Kinet, J. P.; Carpenter, C. L. *Immunity*, **2003**, *19*, 669.
[117] Dombroski, D.; Houghtling, R. A.; Labno, C. M.; Precht, P.; Takesono, A.; Caplen, N. J.; Billadeau, D. D.; Wange, R. L.; Burkhardt, J. K.; Schwartzberg, P. L. *J. Immunol.*, **2005**, *174*, 1385.
[118] Hao, S.; Qi, Q.; Hu, J.; August, A. *FEBS Lett.*, **2006**, *580*, 2691.
[119] Mukhopadhyay, S.; George, A.; Bal, V.; Ravindran, B.; Rath, S. *J. Immunol.*, **1999**, *163*, 1786.
[120] Minoprio, P.; Coutinho, A.; Spinella, S.; Hontebeyrie-Joskowicz, M. *Int. Immunol.*, **1991**, *3*, 427.
[121] Hoerauf, A.; Solbach, W.; Lohoff, M.; Rollinghoff, M. *Int. Immunol.*, **1994**, *6*, 1117.

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4

CONCLUSIONS

4.1 Potential crosstalks of the Tec kinase Itk with the TCR signaling molecules Vav1 and Cbl-b

TCR/CD28 triggering by antigen presenting cells induces a signaling cascade that leads to the activation of signaling molecules, the reorganization of the actin cytoskeleton and the formation of the so-called immunological synapse at the T cell/APC interphase. Together this leads to the activation of downstream signaling components and activation of transcription factors resulting in the induction of IL-2 expression, proliferation of the T cells and the generation of activated effector T cell populations, which is important for the development of a proper adaptive immune response (1, 2). Existing crosstalks between the guanine nucleotide exchange factor Vav1 and the Tec kinase Itk and the similar phenotype of *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ T cells suggest that these two proteins act in the same signaling pathways and at least partially perform similar functions in activated T cells. During my PHD study I further investigated potential interdependences and similarities between Itk and Vav1.

To investigate whether the defects in peripheral *Vav1*$^{-/-}$ and *Itk*$^{-/-}$ T cells are mechanistically similar, we tested whether the defect of *Itk*$^{-/-}$ T cells is rescued upon deletion of the E3 ubiquitin ligase Cbl-b, as described for *Vav1*$^{-/-}$ T cells (3). The analysis of *Itk*$^{-/-}$*Cbl-b*$^{-/-}$ mice revealed partially restored T cell function if *Itk*$^{-/-}$ T cells are released from the negative regulation mediated by Cbl-b. Thus, our data provide additional evidence that Vav1 and Itk fulfill similar functions in peripheral T cells. In addition, we wanted to address genetically whether there is functional redundancy between Vav1 and Itk during T cell activation by generating Vav1/Itk double-deficient mice. The analysis of *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice showed severely reduced numbers of peripheral T cells, most likely as a consequence of diminished numbers of DP cells. *Vav1*$^{-/-}$*Itk*$^{-/-}$ deficient DP cells showed a reduced life span *in vivo* compared to WT and single knock out DP cells, indicating that the combined activity of Itk and Vav1 is necessary for the survival of DP cells. The deletion of

Vav1 and Itk together could alter intracellular signaling pathways that are required for the survival of DP cells. Whether this survival signals are transmitted via cytokine receptors or via the T cell receptor or both still has to be determined. Preliminary data have shown that a kinase-dead version of Itk was able to rescue the survival defect of DP cells in a bone marrow reconstitution experiment, suggesting a scaffolding function for Itk for the survival of DP cells.

The severely reduced numbers of peripheral T cells most likely are a consequence of the reduced numbers of DP cells in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice. However, the remaining *Vav1*$^{-/-}$*Itk*$^{-/-}$ deficient DP cells showed also a defect in positive selection, suggested by a further reduction of Ca^{2+} response in DP cells and the absence of $CD69^{+}$ single positive thymocytes. The already mentioned bone marrow reconstitution experiment showed that the kinase-dead Itk was not able to rescue the positive selection defect in *Vav1*$^{-/-}$*Itk*$^{-/-}$ mice, showing the importance of the kinase function during positive selection, suggesting differential functional activity of Itk during T cell development (illustrated in Fig. 1).

Thus our data indicate an essential role for the combined activity of Vav1 and Itk for the survival of DP thymocytes and positive selection during T cell development.

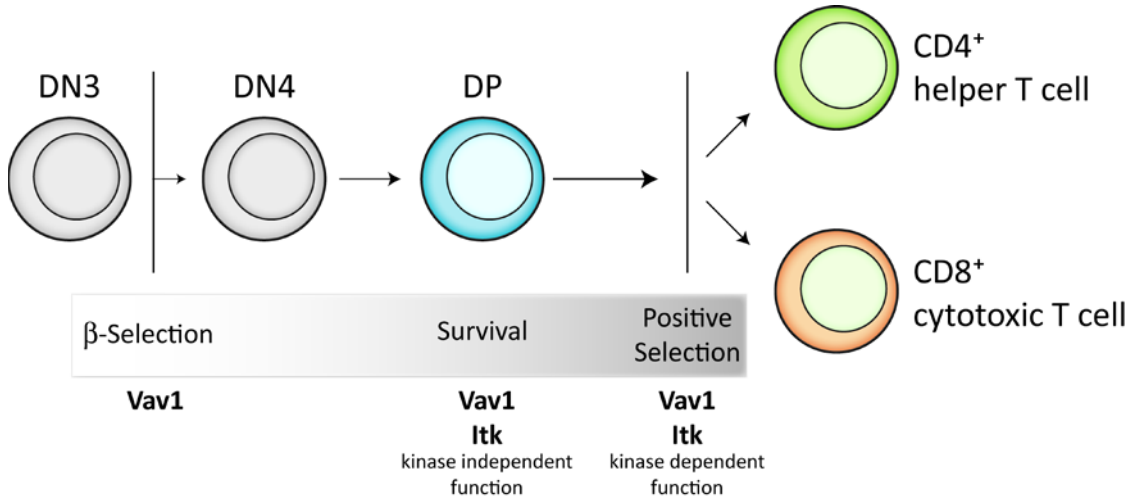

Fig. 1 is illustrating the differential functional activity of the Tec kinase Itk during T cells development together with the guanine nucleotide exchange factor Vav1.

4.2 The transcription factor PLZF and the development and function of memory phenotype T cells

The $CD44^{hi}CD62L^{-}$ memory T cell population consists not only of "true" antigen-specific memory cells that developed in response to a foreign antigen, but includes as well a variety of additional T lymphocyte subsets. These additional cells were described as memory-phenotype (MP) T lymphocytes, and some subsets of them also as innate-like T cells. MP innate-like T cells such as NKT cells, CD8αα IELs or others may play an important role in the front-line defense against certain bacterial infections. NKT cells have also been implicated in regulating autoimmunity and inflammatory diseases, and they have been linked to the immune responses against tumors. Thus, MP innate-like T cells have important immune regulatory functions.

The observations that PLZF is up-regulated in the *Itk–/–* T cell population (which is enriched in innate-like T cells), and that PLZF expression is restricted to the $CD4^{+}CD44^{hi}$ population revealed a correlation between PLZF expression and MP $CD4^{+}$ T cells. During my PHD thesis I wanted to further address a potential role of PLZF in the development and function of memory phenotype T cells by generating T cell specific PLZF transgenic mice. Enforced expression of PLZF during T cell development and in peripheral T cells led to the appearance of a large population of peripheral T cells with a $CD44^{hi}$ memory-phenotype that were able to produce IFNγ upon *ex vivo* PMA/ionomycin stimulation. PLZF expressing (GFP^{+}) T cells in female mice of line #3 (a line with a X-chromosomal integration site, resulting in mosaic expressing females) showed a MP but also similar numbers of GFP^{-} T cells with a naïve phenotype, indicating a T cell intrinsic defect. In addition, the changes in the naïve versus memory-like subset distribution were already present at the SP stage during T cell development, revealing that the MP T cell populations in PLZF transgenic mice are at least in part derived from DP thymocytes, like other MP T cells (4, 5). Moreover, upon anti-CD3/CD28 activation we observed reduced cytokine production in all subsets of PLZF transgenic $CD4^{+}$ T cells compared to WT $CD4^{+}$ T cell subsets, except for the dramatic increase of IL-4 production in the $CD44^{hi}CD62L^{+}$ subset. PLZF expression possibly could lead to a different cellular composition of peripheral $CD4^{+}$ T cells, even though we could not observe any altered expression of T-bet and Gata-3 in the various subsets of PLZF transgenic mice compared to WT mice. These data revealed an important function of PLZF in the development and/or maintenance of memory phenotype T cells.

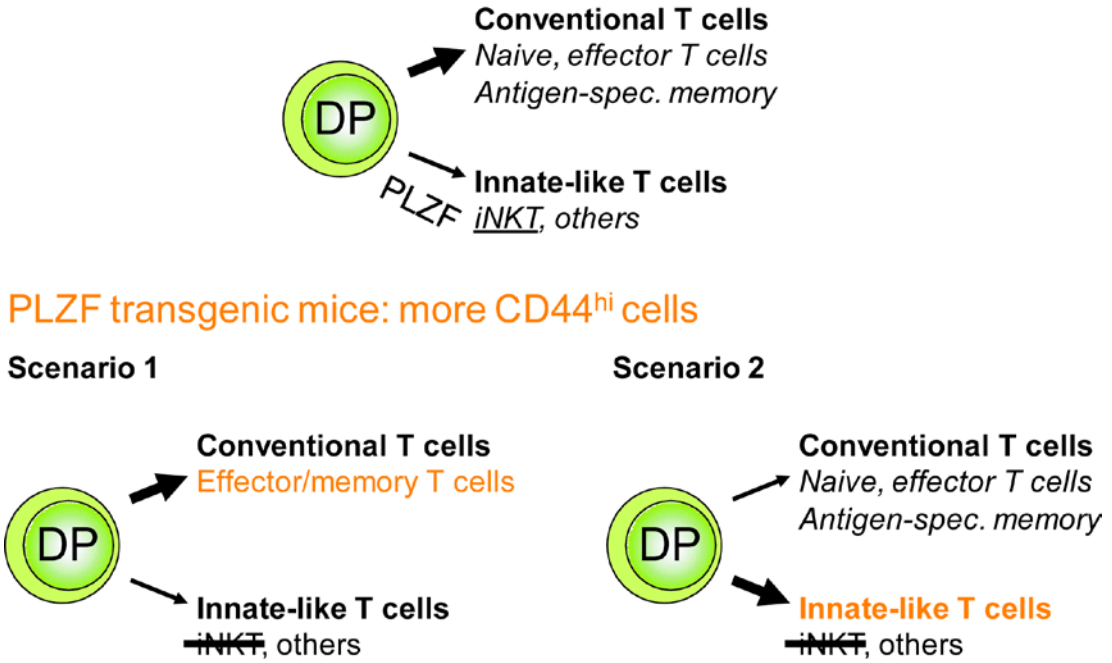

Fig. 2 shows a hypothetic model of PLZF function during T cell development, and a potential role in the regulation of conventional verus non conventional T cell development. In the wildtype situation just very few innate-like T cells develop from a DP progenitor cell (upper panel), but if PLZF expression is enforced in DP and single positive cells, it shifts the balance from a naïve conventional T cell development into the development of either conventional T cells with effector function (scenario 1) or into innate-like T cells (scenario 2).

Two recent studies have shown that PLZF is primarily expressed in CD1d-restricted NKT cells (6, 7). However, other MP T cell subsets may express also PLZF since PLZF expression was upregulated in *Itk–/–* T cells which have diminished numbers of NKT cell subsets compared to WT T cells (8, 9). Interestingly, NKT cell numbers in mice lacking PLZF were severely reduced and they displayed also impaired characteristics usually associated with NKT cells (6, 7). Unexpected to this essential role of PLZF for NKT cell development and function, we observed a severe reduction of NKT cells in PLZF expressing mice compared to WT mice. One explanation that both PLZF-deficient and PLZF transgenic mice have diminished numbers of NKT cells may come from the observation that stage 1 and stage 2 NKT cells express the highest level of PLZF and downregulate PLZF expression at stage 3 (6, 7). Therefore maintained transgenic expression of PLZF during NKT cell development may partially block NKT cells at this stage.

The already mentioned studies by Kovalovsky *et.al.* and Savage *et.al.* demonstrate clear evidence, that PLZF has a unique function in the development of NKT cells and their acquisition of "innate-like" characteristics. In addition our study indicates a more general role for PLZF in decisions made during T cell development between conventional versus innate T cell lineages. In the

transgenic mouse model we could observe the appearance of a large population of CD44^{hi} memory phenotype (MP) T cells beside the NKT cell lineage. So far not much is known about these additional MP T cell lineages. Recent findings from our laboratory are further strengthening the hypothesis that PLZF could be implicated also in the development or function of non-NKT- CD44^{hi} T cells. Semi-quantitative RT-PCR experiments in wildtype T cell subsets revealed, that in addition to NKT cells PLZF is expressed also in other CD44^{hi} CD4^{+} T cell subsets (unpublished data, J.R. and W.E.). The identity of these additional MP T cells still remains to be determined.

There are at least two possibilities to explain the appearance of CD44^{hi} T cells in PLZF transgenic mice. PLZF could convert conventional T cells into effector T cells, or PLZF could be part of a transcription factor network that induces a genetic program that leads to the differentiation of CD44^{hi} innate-like MP T cells (Fig.2). Although we cannot formally exclude the former possibility, some observations argue in favor for the possibility that PLZF (in part) induces an innate-like T cell program. We observed changes in the CD62L expression pattern already in the thymus in CD4SP cells, in agreement with a published study (7). Furthermore, CD8SP cells have already a characteristic innate-like CD44^{hi}CD62L^{lo} expression pattern. This indicates that SP thymocytes are already altered, suggesting developmental changes. Furthermore, ectopic expression of PLZF leads to an increase of CD44^{hi}CD62L^{+} and CD44^{hi}CD62L^{-} T cell subsets that in WT cells express PLZF, suggesting that PLZF can convert CD4^{+}CD44^{lo} subsets into CD4^{+}CD44^{hi} populations and that there is a link between PLZF expression and CD44^{hi} cells. In contrast, reactivated Th1/Th2 cells generated from naïve (CD44^{lo}CD62L^{+}) CD4^{+} T cells did not express detectable levels of PLZF (preliminary data not shown), indicating that effector differentiation is not linked with PLZF expression. We further demonstrated that CD4^{+} and CD8^{+} MP T cells in PLZF mice possess in part innate-like characteristics. The increase in IFNγ-producing transgenic CD44^{hi} CD8^{+} T cells is similar to the one observed in innate-like *Itk–/–* CD8^{+} T cells, although the percentage of IFNγ-positive cells within the CD44^{hi} population is higher in the absence of Itk. However, only peripheral MP CD8^{+} T cells and not CD8SP MP thymocytes in PLZF transgenic mice showed an increase in IFNγ-producing CD44^{hi} cells upon PMA/ionomycin stimulation, potentially suggesting peripheral maturation processes of innate-like functions in CD8^{+} PLZF transgenic T cells. In contrast, IFNγ-producing CD44^{hi} cells were increased in the thymus and in the periphery in *Itk–/–* mice. Thus, only certain features are shared between PLZF transgenic T cells and the innate-like T cell subsets described in *Itk–/–* mice. It is not known whether innate-like *Itk–/–* T cell populations have the same subset composition as WT innate-like T cell populations, and whether they function in

the same way like wt innate-like T cells. Therefore, differences between innate-like T cells from *Itk–/–* and PLZF transgenic mice may not be surprising.

To determine whether, and if so how PLZF could regulate the decision between innate and conventional T cells further studies are required. One interesting question could address the timing of PLZF expression during T cell development. It would be interesting to determine whether PLZF has to be expressed at a certain developmental stage (e.g. during positive selection) or whether there is a developmental window at which PLZF expression is required to induce the appearance of memory phenotype T cells. Furthermore it would be interesting to identify the signals and factors which induce the expression of PLZF. An additional interesting approach would be the identification of PLZF target genes by e.g. using Chip on ChIP technologies.

There are still many open questions to be addressed concerning PLZF and its possible role during memory phenotype T cell development and function. The answers will give important insight into the development and function of non conventional T cells and therefore provide a better understanding of the first line of the adaptive immune system in the fight against an infection.

4.3 References

1. Samelson, L.E. 2002. Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. *Annu Rev Immunol* 20:371-394.
2. Davis, D.M., and M.L. Dustin. 2004. What is the importance of the immunological synapse? *Trends Immunol* 25:323-327.
3. Krawczyk, C., K. Bachmaier, T. Sasaki, R.G. Jones, S.B. Snapper, D. Bouchard, I. Kozieradzki, P.S. Ohashi, F.W. Alt, and J.M. Penninger. 2000. Cbl-b is a negative regulator of receptor clustering and raft aggregation in T cells. *Immunity* 13:463-473.
4. Surh, C.D., O. Boyman, J.F. Purton, and J. Sprent. 2006. Homeostasis of memory T cells. *Immunol Rev* 211:154-163.
5. Berg, L.J. 2007. Signalling through TEC kinases regulates conventional versus innate CD8(+) T-cell development. *Nat Rev Immunol* 7:479-485.
6. Kovalovsky, D., O.U. Uche, S. Eladad, R.M. Hobbs, W. Yi, E. Alonzo, K. Chua, M. Eidson, H.J. Kim, J.S. Im, P.P. Pandolfi, and D.B. Sant'Angelo. 2008. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol* 9:1055-1064.

7. Savage, A.K., M.G. Constantinides, J. Han, D. Picard, E. Martin, B. Li, O. Lantz, and A. Bendelac. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29:391-403.
8. Au-Yeung, B.B., and D.J. Fowell. 2007. A key role for Itk in both IFN gamma and IL-4 production by NKT cells. *J Immunol* 179:111-119.
9. Felices, M., and L.J. Berg. 2008. The tec kinases itk and rlk regulate NKT cell maturation, cytokine production, and survival. *J Immunol* 180:3007-3018.
10. Dave, V.P., D. Allman, R. Keefe, R.R. Hardy, and D.J. Kappes. 1998. HD mice: a novel mouse mutant with a specific defect in the generation of CD4(+) T cells. *Proc Natl Acad Sci U S A* 95:8187-8192.
11. Sun, G., X. Liu, P. Mercado, S.R. Jenkinson, M. Kypriotou, L. Feigenbaum, P. Galera, and R. Bosselut. 2005. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. *Nat Immunol* 6:373-381.
12. He, X., X. He, V.P. Dave, Y. Zhang, X. Hua, E. Nicolas, W. Xu, B.A. Roe, and D.J. Kappes. 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature* 433:826-833.
13. Wang, L., K.F. Wildt, J. Zhu, X. Zhang, L. Feigenbaum, L. Tessarollo, W.E. Paul, B.J. Fowlkes, and R. Bosselut. 2008. Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. *Nat Immunol* 9:1122-1130.
14. Egawa, T., and D.R. Littman. 2008. ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. *Nat Immunol* 9:1131-1139.
15. Muroi, S., Y. Naoe, C. Miyamoto, K. Akiyama, T. Ikawa, K. Masuda, H. Kawamoto, and I. Taniuchi. 2008. Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. *Nat Immunol* 9:1113-1121.

Julia Raberger
Neumayrgasse 18/16, 1160 Wien
Mobil: +43 (0) 650 2080380
E-mail: Julia.raberger@meduniwien.ac.at

Curriculum Vitae

Personal data	Date of birth	08.03.1980
	Place of birth	Wels
	Family status	not married
	Education	Mag.rer.nat. Genetics/Microbiology
	Special subject	Immunology

Education	since 2005	PhD study in genetics/microbiology at the University of Vienna PhD thesis subject: immunology „Tec kinases in T cell development and T cell receptor signaling”
	1999 – 2005	Master study in genetics/microbiology with distinction at the University of Vienna Master thesis subject: cell biology “Studien zur Interaktion des Cytolinkerproteins Epiplakin mit Keratinfilamenten“
	1994 – 1999	High school for chemical engineering (HTBLA) in Wels

Extra-curricular activities	2002-2003	Technician at the Max F. Perutz Laboratories (MFPL) Medical University Vienna Genotyping of mouse strains, DNA-Isolations, Polymerase Chain Reaction (PCR), Acquisition and analysis of data

Julia Raberger
Neumayrgasse 18/16, 1160 Wien
Mobil: +43 (0) 650 2080380
E-mail: Julia.raberger@meduniwien.ac.at

Curriculum Vitae

Technical skills	Biochemistry	SDS-PAGE, Western blot, Immunoprecipitation, Expression and Purification of recombinant proteins, Purification of polyclonal antibodies
	Tissue Culture	Cultivation and isolation of $CD4^+$ -, $CD8^+$- T cells and keratinocytes, Transfections and infections (transient gene transfer) of cell lines and primary cells
	Molecular biology	Molecular cloning, Vector subcloning, PCR, cDNA, Semiquantitative RT-PCR, Realtime
	Immunolgy/cell biology	ELISA, Immunofluorescence stainings, Confocal microscopy, Intracellular cytokine stainings, FACS (Calibur, LSRII, BD Bioscience) T cell proliferations, T cell polarizations
Additional skills	Language	English (fluent)
	Teaching experience	Supervison of Master students Tutorials of several practical courses at the University of Vienna
	Public relations	Presentation of scientific results at national and international conferences Publications in Scientific Journals Organization of the SFB Students- Symposium 2007 (Dentritic cells and T cells at the interface of innate and adaptive immunity)
	Additional qualifications	REFA-Grundschein

Julia Raberger
Neumayrgasse 18/16, 1160 Wien
Mobil: +43 (0) 650 2080380
E-mail: Julia.raberger@meduniwien.ac.at

Curriculum Vitae

IT skills	general	MS Office, Adobe Photoshop, Adobe Illustrator and Adobe InDesign
	Acquisition/Analysis Programs	Flowjo (Tristar), Cellquest Pro (BD Bioscience) and Graphpad Prism

Publications:

Raberger J, Schebesta A, Sakaguchi S, Boucheron N, Blomberg K. E, Berglöf A, Kolbe T, Smith C.I. E, Rülicke T, Ellmeier W.
The Transcriptional Regulator PLZF Induces the Development of CD44-High Memory-Phenotype T Cells. Proc Natl Acad Sci U S A. 2008 Nov 12

Raberger J, Boucheron N, Sakaguchi S, Penninger J. M, Ellmeier W.
Impaired T cell development in the absence of Vav1 and Itk. Eur J Immunol. 2008 Nov 13

Spazierer D, Raberger J., Gross K, Fuchs P, Wiche G.
Stress-induced recruitment of epiplakin to keratin networks increases their resistance to hyperphosphorylation-induced disruption. J Cell Sci. 2008 Mar 15;121(Pt 6):825-33

Melcher M, Raberger J, Schmidt U, Unger B, Ellmeier W.
The Role of Tec family kinases in inflammatory processes. Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry, 2007, 6, 61-69