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Gene expression analysis of Tec family kinases in B- and T-lymphocytes

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ABSTRACT

The microarray technology is a powerful tool used in many different research areas. The technique has been around for more than a decade and has revolutionized the molecular biology field. In this thesis the Affymetrix GeneChip® arrays were used to study the transcriptomes of Tec family kinase mutant mouse models. Bruton's tyrosine kinase (Btk), IL-2 inducible T-cell kinase (Itk) and Tyrosine kinase expressed in hepatocellular carcinoma (Tec) are protein tyrosine kinases belonging to this Tec kinase family. Mutations in Btk cause a primary immunodeficiency disease called X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency disease (Xid) in mice. Gene expression profiling was performed on whole splenic B-cells as well as Transitional 1 (T1) B-cells from Xid, Btk knockout (Btk KO) and control mice. This was done in order to study differences and similarities between Btk-defective mice (Xid and Btk KO together) compared to control. Small differences were distinguished between the Btk-defective mouse strains in the whole splenic B-cell population; only seven genes differed (>2-fold) between the two. A number of potentially interesting genes were found to be differentially expressed in the Btk-defective groups compared to the controls. We also show that the Btk defect is already manifested at the T1 B-cell stage.

Itk is important for the T-cell development where it has a role in regulating the conventional versus innate T-cells. Itk-deficient T-cells are of an innate, memory-like type. Tec is poorly expressed in resting T-cells, but is expressed 2-3 days after stimulation. Tec-deficient mice show no phenotypic alterations and the mice develop normally. In Papers III and IV we looked at the transcriptomes of Itk-, Tec- and Itk/Tec-deficient mice by studying unstimulated as well as stimulated $CD3^+$ T-cells. The Itk-deficiency was also studied in $CD4^+$ and $CD8^+$ T-cell subpopulations. The gene expression patterns of Tec-deficient mice compared to control mice showed small differences, further supporting earlier findings. More differences were seen in Itk-deficient as well as Itk/Tec-deficient mice compared to controls. We also investigated if the Itk-deficiency could mimic calcineurin inhibition by treating Wt T-cells with cyclosporin A (CsA). CsA was shown to have a stronger effect on transcriptional regulation than Itk-deficiency, suggesting that only a fraction of TCR-mediated calcineurin/NFAT-activation is dependent on Itk. Greater differences were seen in $CD4^+$ and $CD8^+$ T-cells in comparison to total $CD3^+$ T-cells. In Paper IV we found

Zbtb26, an interesting transcription factor, being up-regulated in Itk-deficient T-cells, while normalized in Itk/Tec-deficient cells compared to Wt. By the use of a combination of the microarray technology and quantitative RT-PCR analysis, we have evaluated the transcriptomes of Btk-, Itk-, Tec- and Itk/Tec-deficient mice.

LIST OF PUBLICATIONS

This thesis is based on the following articles and manuscript, which will be referred to in the text by their Roman numerals.

I. Lindvall JM, **Blomberg KEM**, Berglöf A, Yang Q, Smith CIE and Islam TC.
Gene expression profile of B cells from Xid mice and Btk knockout mice.
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II. Lindvall JM, **Blomberg KEM**, Berglöf A and Smith CIE.
Distinct gene expression signature in Btk-defective T1 B-cells.
Biochem Biophys Res Commun 2006 Jul 28;346(2):461-9.

III. **Blomberg KEM**, Boucheron N, Lindvall JM, Yu L, Raberger J, Berglöf A, Ellmeier W and Smith CIE.
Transcriptional signatures of Itk-deficient $CD3^+$, $CD4^+$ and $CD8^+$ T-cells. *BMC Genomics in press.*

IV. **Blomberg KEM**, Boucheron N, Lindvall JM, Vargas L, Raberger J, Berglöf A, Ellmeier W and Smith CIE.
Tec and Itk regulate overlapping signaling pathways in T lymphocytes.
Manuscript

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Raberger J, Schebesta A, Sakaguchi S, Boucheron N, **Blomberg KEM**, Berglöf A, Kolbe T, Smith CIE, Rülicke T, Ellmeier W.
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* These authors contributed equally to this paper

CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
bp	Base pair
BCR	B-cell receptor
BH	Btk homology
BLNK	B-cell linker protein
Bmx/Etk	Bone Marrow kinase gene on the X-chromosome/Epithelial and endothelial tyrosine kinase
BTB-ZF	Bric-a-brac, tramtrack, broad complex domain-containing family of zinc finger proteins
Btk	Bruton's tyrosine kinase
Btk KO	Btk knockout
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CsA	Cyclosporin A
dChip	DNA-Chip Analyzer
DN	Double negative
DP	Double positive
EBF1	Early B cell factor 1
EBV	Epstein-Barr-Virus
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signaling kinase
EST	Expressed Sequence Tag
FDR	False discovery rate
FO B-cell	Follicular B-cell
FRET	Fluorescence resonance energy transfer
GATA3	GATA-binding protein 3
GC	Germinal center
GCOS	Affymetrix GeneChip® Operating Software
GO	Gene Ontology
Iba1	Ionized Ca^{2+} binding adapter molecule 1
IFN-γ	Interferon-γ
Ig	Immunoglobulin
Igf1	Insulin-like growth factor 1
IL	Interleukin
IP_3	Inositol (1,4,5) triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
Itk	IL-2 inducible T cell kinase
IVT	In vitro transcription
Klr	Killer cell lectin-like receptor
MAIT	Mucosal-associated invariant T-cells
MAPK	Mitogen-associated protein kinase
mEH/Ephx1	Microsomal epoxide hydrolase
MHC	Major Histocompatibility Complex
MM	Mismatch

MZ	Marginal zone
NFAT	Nuclear Factor of Activated T-cells
NF-κB	Nuclear factor kappa B
NKT	Natural killer T-cells
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein Kinase B (also known as Akt)
PLCγ	Phospholipase C gamma
PLZF	Promyelocytic leukemia zinc finger (the same as Zbtb16)
PM	Perfect match
Pre-B-cell	Precursor B-cell
PRR	Proline rich region
Rlk/Txk	Resting lymphocyte kinase/T and X cell expressed kinase
RMA	Robust Multi-array Average
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcriptase PCR
SAP	SLAM-associated protein
SH	Src homology
SH2D1A	SH2 domain protein 1A
SLAM	Signaling lymphocytic activation molecule
SNP	Single Nucleotide Polymorphism
SOM	Self-organizing map
Tc	Cytotoxic T-cell
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl transferase
Tec	Tyrosine kinase expressed in hepatocellular carcinoma
TH	Tec homology
Th	T helper cell
TNF-β	Tumor necrosis factor β
TLR	Toll-like receptor
T1 B-cell	Transitional type 1 B-cell
T2 B-cell	Transitional type 2 B-cell
Wt	Wild type
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia
XLP	X-linked lymphoproliferative syndrome
ZAP-70	ζ chain-associated protein kinase of 70 kDa
Zbtb16	Zinc finger and BTB domain containing 16
Zbtb26	Zinc finger and BTB domain containing 26

1 INTRODUCTION

Gene expression profiling using microarrays has become a huge research area today. The technique has enabled researchers to simultaneously analyze thousands of genes in a single experiment. The microarray technology is based on the principle of hybridization between two complementary strands of nucleic acids, one being fixed into a solid membrane, the other being the sample to analyze. A microarray is comparative to a chip or slide with a lot of small spots on it. The spots are called probes and they consist of short gene-specific sequences that will bind to a fluorescently labeled nucleic acid sequence, called target, through complementary base-pairing. Targets could be either DNA or RNA from biological samples. If the target hybridizes to the probe you will be able to measure a hybridization signal. There are numbers of different applications for microarrays, e.g. determine specific gene patterns in a certain type of cancer or study the effect of different treatments.

In this thesis I have used gene expression profiling for determining similarities and differences in the transcriptomes of Tec family kinase mutant mouse models.

1.1 TEC FAMILY KINASES

1.1.1 Protein structure

The Tec family of non-receptor protein-tyrosine kinases consists of five members; they are Bmx (Etk), Btk, Itk, Txk (also known as Rlk) and Tec [1]. They are called tyrosine kinases due to their ability to phosphorylate their substrates on tyrosine residues. The family has been described in mammals and birds, but recently a Tec family kinase ancestor was found to exist in the unicellular choanoflagellate *Monosiga brevicollis* [2]. The particular domains of Tec kinases are the Pleckstrin homology (PH) domain and the Tec homology (TH) domain at their N-terminus. The PH domain is important for membrane translocation, and the TH domain is specific for the Tec family of kinases. It contains a Zn^{2+}-binding Btk homology (BH) motif and also one or two proline-rich regions (PRR). Bmx does not encompass any PRRs. All members of the family except Rlk/Txk have the N-terminal PH domain, but Rlk/Txk is yet classified to the Tec family. After the TH domain follows three other domains called Src homology (SH) domains; SH1, SH2 and SH3 (see Figure 1). SH1 is also called the kinase domain

because of its catalytic activity. The SH2 domain has a pocket that binds phosphotyrosine-containing peptide motifs. The SH3 domain binds to proline-rich segments with the consensus sequence PXXP (X is any amino acid) [3]. Rlk/Txk has an N-terminal palmitoylated cysteine-rich sequence instead of a PH domain. That sequence anchors Rlk/Txk to the membrane and makes it constitutively localized there [4].

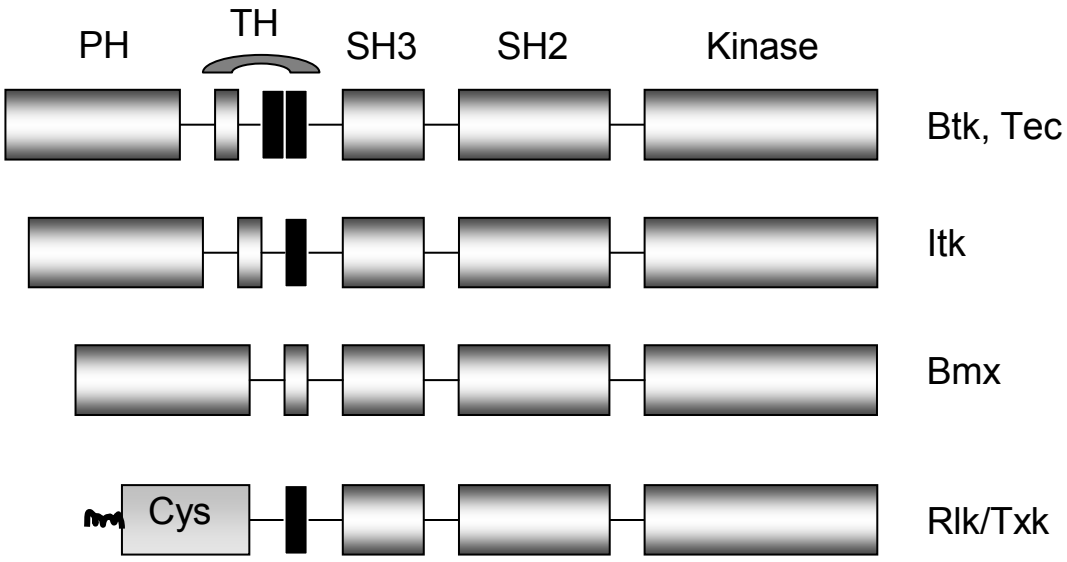

Figure 1. The different domains of the Tec family kinases. The TH domain is composed of two parts; the most N-terminal is the BH motif (grey) and the black boxes are the PRRs. Rlk/Txk has instead of a PH domain a palmitoylated cysteine-rich sequence.

1.1.2 Expression of Tec family kinases

The Tec kinase family is mainly expressed in cells of the hematopoietic system, mostly in B- and T-lymphocytes, except for Bmx and Tec, which are more broadly expressed [1, 5, 6]. Each Tec kinase has its own expression pattern described in more detail below.

Bmx was initially identified in bone marrow and subsequently in epithelial cells, fibroblasts, and endothelial cells [7-10]. Tec has two major splice forms, Tec III and Tec IV, which are differentially expressed in thymus, bone marrow, endothelial and hematopoietic cells and melanocytes [1]. Btk and Tec are primarily expressed in B-cells [11], while T-cells express Itk, Tec and Rlk/Txk. Itk is the highest expressed

family member in T-cells and is induced after T-cell activation. Tec is the lowest expressed. However, Tec is induced after 2-3 days of T-cell receptor (TCR) stimulation suggesting a role for Tec in T-cell effector function and restimulation [12]. The expression of Rlk/Txk is intermediate the expression of Itk and Tec in T-cells, and after TCR stimulation its expression rapidly decreases.

This family of kinases is also expressed in the myeloid lineage such as in macrophages (Bmx, Btk and Tec) and in neutrophils (Bmx, Btk and Tec), but also in mast cells (Btk, Itk and Tec), platelets (Btk and Tec) and dendritic cells (Btk) [6].

1.2 B-CELL DEVELOPMENT

B-cell development begins in the fetal liver and continues in the bone marrow throughout our lives. The development is characterized by two phases; the first phase occurs in the bone marrow and is antigen-independent and the second phase is when the immature B-cell leaves the bone marrow and is ready to encounter antigen (antigen-dependent phase) [13]. B-cells arise from hematopoietic stem cells that will differentiate into a progenitor B-cell (pro-B-cell) (Figure 2). The pro-B-cells require contact with bone-marrow stromal cells for further maturation. This contact is mediated by several cell-adhesion molecules such as VLA-4 on the pro-B-cell and VCAM-1 on the stromal cell. c-kit is a receptor on the pro-B-cell which binds to stem cell factor (SCF) on the stromal cell surface and mediates further development into precursor B-cells (pre-B-cells). The stromal cells also produce interleukin 7 (IL-7) that is important for the pre-B-cell [13]. The development continues with the immature B-cell, which expresses membrane Immunoglobulin M (mIgM) on its cell surface. During the stages in the bone marrow the Ig gene rearrangement occurs resulting in one productive heavy-chain gene and one productive light-chain gene that in the end will function as the B-cell receptor (BCR). The immature B-cells can be divided into Transitional type 1 (T1) and type 2 (T2) B-cells. The antigen-dependent phase begins once the immature B-cell migrates to the peripheral lymphoid tissue such as lymph node and spleen. Immature B-cells that express auto-antibodies against self-antigens will be negatively selected and eliminated. Then it evolves into a mature (naïve) B-cell that expresses IgM and IgD at its surface.

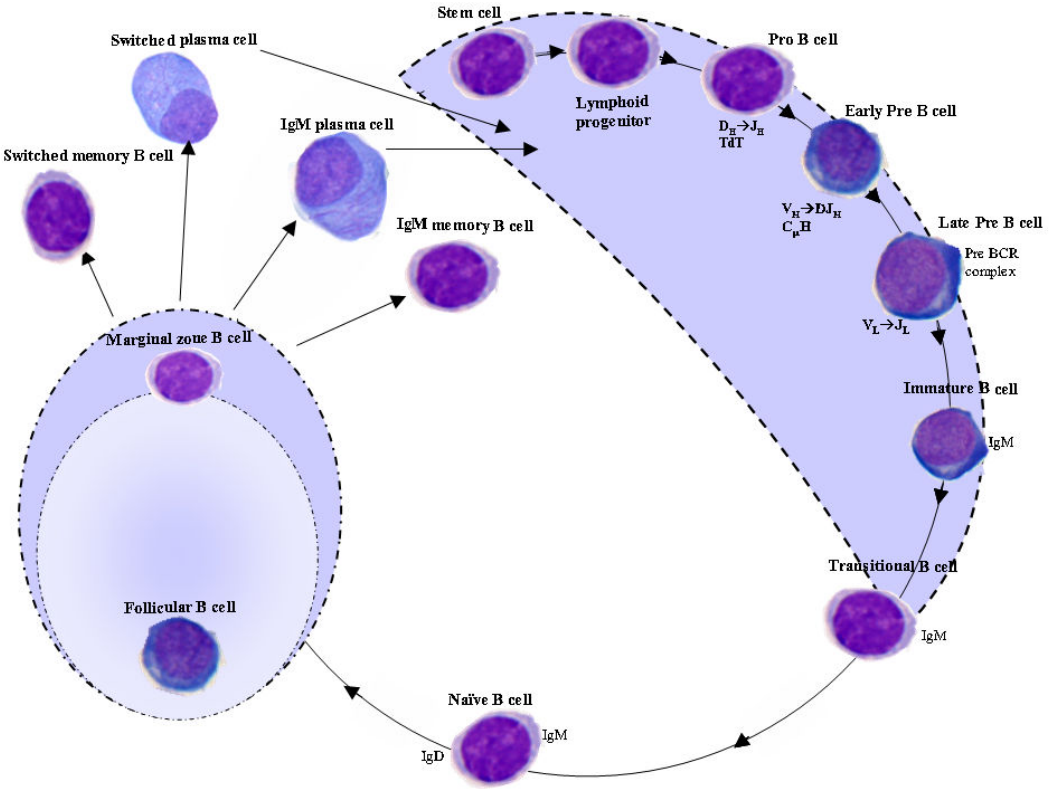

Figure 2. B-cell development from bone marrow to secondary lymphoid organs.

Mature B-cells recirculate among the lymphoid follicles of spleen and lymph nodes. B-2 (also known as follicular (FO) B-cells) and marginal zone (MZ) B-cells are two main types of peripheral B-cells [14]. FO B-cells cooperate with T-cells to generate the adaptive immune response. MZ B-cells reside in close proximity to the marginal sinus region of the spleen that filters blood, which is in a good position to detect foreign antigens [15]. When MZ B-cells recognize antigens they respond rapidly by proliferating and differentiating into plasma cells and this is done mostly independent of T-cell help [16]. These MZ B-cells are short-lived and generate low-affinity antibodies. They represent a first line of defense against pathogens [17, 18]. The T-cell dependent immune responses take longer time to become established and here the FO B-cells have an important role. They are found in secondary follicles and form germinal centers (GCs) in lymph nodes and spleen. The FO B-cells present antigen to T-cells and generate high-affinity antibody responses and are the long-term main players in plasma-cell production and memory B-cell generation [18].

A third type of mature peripheral B-cell is the B-1 B-cell. It is mainly derived from fetal liver stem cells and is primarily found in the peritoneal cavity and some in the spleen [13, 19]. It expresses high levels of IgM and low levels of IgD as well as CD11b/Mac-1, which is a surface marker also found on macrophages and granulocytes [20]. B-1 B-cells can be further subdivided into B-1a ($CD5^+$) and B-1b ($CD5^-$) B-cells [21]. B-1a cells populate the peritoneal and pleural cavity and are here responsible for the non-adaptive first line of defense by secreting "natural antibodies", predominantly of the IgM class, that bind to a variety of T-independent antigens [22-24].

1.2.1 Transcription factors involved in B-cell development

The production of B-cells occurs in the bone marrow, but before the decision that a cell is going to be a B-cell, a controlled process involving cytokine receptors and transcription factors occurs. Early B-cell development and lineage commitment critically depends on the activity of two growth factor receptors, the IL-7R and Flt3, as well as a number of transcription factors, including Ikaros, PU.1, E2A, early B cell factor 1 (EBF1) and Pax5 that function in a transcriptional network [25]. One of the suggested key players of early B-cell development is EBF1 [26, 27], which is regulated by E2A. If EBF1 is missing, a complete block of peripheral B-lymphoid cells occurs [26]. One study has even found that EBF1-deficient mice show a B-cell developmental block at the common lymphoid progenitor (CLP) stage [28].

E2A is a so called E protein together with HEB and E2-2 [29]. E2A can be alternatively spliced into the two E proteins E12 and E47. The function of E2A is inhibited by the transcription factor Notch, which inhibits the E47 protein resulting in an impairment of B-cell development [30]. Notch usually promotes T-cell development. Also, EBF1 is affected by Notch signaling. One paper shows that Notch signaling inhibits the EBF1 activity as well as the coordinated activity of EBF1 and E2A [31].

The role of Pax5 has been extensively studied and by analyzing Pax5-deficient pro-B cells a dual role for Pax5 in lymphopoiesis has been observed. It activates genes associated with the B-cell lineage while at the same time repressing genes involved in other hematopoietic cell lineages [32, 33]. Pax5 has a function in activated B-cells, but is transcriptionally silenced in plasma cells [34].

1.2.2 Btk activation

Btk is involved in a B-cell's proliferation, development, differentiation, survival and apoptosis [35-38]. Btk has been shown to be a component of multiple signaling pathways, the BCR pathway is the most elucidated [39]. Following BCR engagement, so called immunoreceptor tyrosine-based activation motifs (ITAMs) in the Igα/Igβ tails are phosphorylated by Src family members such as Lyn. Phosphoinositide 3-kinase (PI3K) is activated leading to phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphatidyl-inositol-3,4,5-trisphosphate (PIP_3). Btk´s PH domain will bind to PIP_3 and Btk is recruited to the inner surface of the plasma membrane [40-42]. In the plasma membrane Btk is phosphorylated by Src family kinases at tyrosine 551 (Y551), then follows an autophosphorylation event at Y223 [43, 44]. At the same time the adapter protein B-cell linker (BLNK/SLP-65) is recruited to phosphorylated tyrosines in the Igα tail and there it is phosphorylated by Syk. Phosphorylated BLNK will provide binding sites for Btk and phospholipase Cγ2 (PLCγ2). Btk phosphorylates PLCγ2 which leads to the hydrolysis of PIP_2 into inositol (1,4,5) triphosphate (IP_3) and diacylglycerol (DAG). This will lead to Ca^{2+} mobilization and protein kinase C (PKC) activation [45, 46]. Btk also has a role in nuclear signaling. It was previously shown to shuttle between the nucleus and cytoplasm [47], but the molecular mechanism behind this phenomenon is not understood. Btk stimulates nuclear factor of kappa B (NF-κB) signaling and uses a positive auto-regulatory feedback mechanism to stimulate transcription from its own promoter via NF-κB [48, 49].

Btk is also involved in the interaction with members of the Toll-like receptor (TLR) family. Interactions have been found with TLR4, TLR6, TLR8 and TLR9. This implies that Btk has an important role in the function of immune cells of innate as well as adaptive immunity [50, 51]. The TLR9 ligand CpG can stimulate B-cells to phosphorylate Btk. In the absence of Btk, the TLR9-induced proliferation of B-cells is impaired [52].

1.2.3 Btk-deficiency in humans

Mutations in the *BTK* gene cause a primary immunodeficiency disease called X-linked agammaglobulinemia (XLA) in humans [53]. Patients with XLA have defective B-cell development resulting in an inability to produce antibodies. A mutation causes a partial block at the pro- to the pre-B-cell stage as well as a total block at the mature B-

lymphocyte stage [54, 55]. A vast number of distinct mutations have been described all over the *BTK* gene [56-58]. The disease was originally described in 1952 by Dr. Ogden C. Bruton [59]. Owing to the absence of antibodies the patients suffer from recurrent bacterial infections, in particular bacterial respiratory tract and gastrointestinal infections, but also enteroviral infections [60, 61].

The *BTK* gene is located in the X-chromosome on Xq22.1. Due to the X-linked inheritance only boys are affected. Approximately 1 in 200,000 males is infected. Females that carry a *BTK* mutation in one of their X-chromosomes are healthy and display a non-random X-chromosome inactivation, which is limited to B-cells, and results in selection of the healthy X-chromosome [62]. However, there is a single report on a female that got XLA from her father due to an exclusive inactivation of the maternal X-chromosome [63].

The onset of XLA is within the first year of life, after disappearance of maternal immunoglobulin G (IgG) [60, 64]. A pronounced decrease of all serum Igs are seen [65], together with absence of B-cells as well as plasma cells. The treatment still remains the same as Dr. Bruton developed; a combination of gamma globulin substitution and antibiotics to treat the infections.

1.2.4 Btk-deficiency in mice

The corresponding disease for XLA in mouse is called X-linked immunodeficiency disease (Xid) and is much milder than XLA. This is due to a later and partial B-cell developmental block between the pre-B- and mature B-cell stage [66, 67], resulting in that the mouse has more circulating B-cells compared to the XLA-patients. The Xid mouse carries a single amino acid substitution in the PH domain of Btk, an arginine is substituted into a cysteine at position 28 (R28C) [68, 69]. The R28C mutation has also been shown in humans with classical XLA [58]. Xid mice show reduced levels of IgM and IgG3 antibodies [70], have a loss of peritoneal $CD5^+$ B-1a cells and are hyporesponsive to mitogenic anti-Igs and thymus-independent type II antigens [70-72]. Btk knockout (Btk KO) mice lack detectable protein and have an Xid-like phenotype. Btk-deficient cells have impaired Ca^{2+} mobilization [45, 73].

The milder phenotype in mice could be due to that another Tec kinase compensates for the loss of Btk. Tec has been shown to functionally compensate for Btk in Btk KO mice [74].

1.3 T-CELL DEVELOPMENT

Lymphoid progenitors arise in the bone marrow and leave for the thymus where they develop into double negative (DN) commited T-cell precursors (Figure 3). Those early T-cell precursors lack expression of TCR as well as CD4 and CD8 and can give rise to γδ or αβ TCR-expressing cells [75]. The majority of thymocytes and peripheral T-cells bear αβ TCRs [76]. The DN thymocytes can be divided into four stages of differentiation due to their expression of CD44 and CD25. DN1, $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$ and DN4, $CD44^{-}CD25^{-}$ [77]. Rearrangement of the TCR-β loci occurs at the DN3 stage and later the pre-TCR-α is expressed and results in a pre-TCR during the DN4 stage. The pre-TCR pairs with a complex called CD3/ζ that is involved and important for the signal transduction. Adequate signaling is required for further T-cell maturation and will result in replacement of pre-TCR-α into rearranged TCR-α chain. This, together with expression of the co-receptors CD8 and CD4 will turn them into double positive (DP) thymocytes [75]. The DP thymocyte interacts with cortical epithelial cells expressing Major Histocompatibility Complex (MHC) class I and II molecules with self-peptides [78]. Then follows a test on how much signaling that is mediated by interaction of the TCR and the MHC-presented peptides. Most of the DP thymocytes are short-lived and die because their TCR fails to transduce signals. Thymocytes are negatively selected when their TCRs show high-affinity for the self-peptides. An intermediate level of signaling is selected for (positive selection). Some studies have shown that the positive selection process is dependent on the protein tyrosine kinase Lck, which is bound to CD4 and CD8 with different affinities and therefore determines the outcome of the lineage choice of DP thymocytes [79-82]. Strong Lck signaling seems to favor the differentiation into $CD4^{+}$ T-cells, while little or no signaling results in $CD8^{+}$ T-cells [83]. The extracellular signaling kinase (ERK), which is a component downstream of the TCR, has been shown to promote $CD4^{+}$ T-cell development [84]. Thymocytes that express TCRs that bind to MHC class I peptides will develop into $CD8^{+}$ T-cells, while those that express TCRs binding to

MHC class II peptides will be $CD4^+$ T-cells [85, 86]. After the positive selection the cells are ready to be exported to peripheral lymphoid tissues.

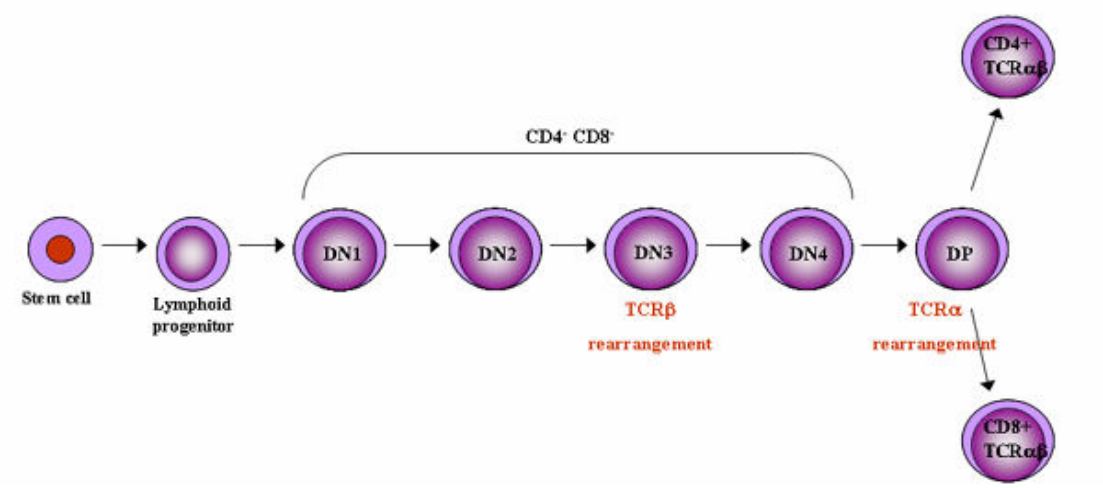

Figure 3. Overview of T-cell development in the thymus.

The $CD4^+$ T-cells are further divided into Th1 and Th2 cells. Th1 cells express interferon-γ (IFN-γ), IL-2 and tumor necrosis factor β (TNF-β) and are responsible for classical cell-mediated functions such as activating cytotoxic T-cells (Tc). Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 and they function more effectively as helpers for B-cell activation. Two transcription factors are important for the Th cell commitment, they are T-bet and GATA-binding protein 3 (GATA3) for Th1 and Th2 cells, respectively [87]. Differences in Tec family kinase expression levels can be seen in the Th cells. Rlk/Txk has been shown to be expressed preferentially in Th1 rather than Th2 cells [88, 89]. However, the situation for Itk is the opposite; increased levels during differentiation into Th2 cells [89]. Also Tec expression is higher in Th2 cells than in Th1 cells [12].

The $CD8^+$ T-cells are called cytotoxic T-cells and are able to eliminate altered self-cells such as virus-infected cells and tumor cells.

1.3.1 Conventional versus innate T-cells

T-cells are divided into conventional and innate T-cells due to the different surface markers they express. Conventional T-cells are the characteristic αβ T-cells with expression of the coreceptors CD4 and CD8, but low expression of CD44 and little to no expression of CD122. γδ T-cells, natural killer T- (NKT) cells, mucosal-associated

invariant T- (MAIT) cells, CD8αα intraepithelial T-cells and H2-M3 restricted T-cells are examples of innate T-cells [90]. They express high levels of CD44, CD122 and NK-cell markers such as NK1.1, all of which are characteristics of an activated or memory phenotype [90]. Innate T-cells constitutively express transcription factors that regulate effector genes; this explains their rapid effector responses after TCR stimulation [91-93]. They have also been shown to be dependent on IL-15 [94]. Their TCRs are specific for non-classical MHC class Ib molecules (CD1d, MR1, Qa-1 and H2-M3) [95] instead of the classical MHC class I and II that conventional T-cells recognize. Still they are selected in the thymus, but through interactions with hematopoietic cells, such as thymocytes or B-cells, rather than epithelial cells [96-99].

Itk and Rlk/Txk have been shown to be required for the development of conventional naïve $CD8^+$ T-cells [100-102].

1.3.2 Itk-deficiency

Itk-deficient mice show higher numbers of $CD8^+$ T-cells than their wild type counterparts. Those $CD8^+$ T-cells are not normal naïve T-cells but of an innate-like type with memory characteristics, such as expression of the activation markers CD44 and CD122 (IL-2Rβ chain), as well as expression of TCRs specific for non-classical MHC class Ia and Ib molecules [100-103]. Expression of CD122 and production of IFN-γ indicate that these cells express a T-box transcription factor such as T-bet or eomesodermin [104]. Both of the factors were shown to be up-regulated in Itk-defective mice [89, 100, 105]. They also rapidly release cytokines upon TCR engagement [90, 100, 101]. Itk-deficient mice show impaired positive selection of $CD4^+$ T-cells and it was suggested that Itk modulates signaling thresholds during T-cell development [106-108]. It was also shown that Itk-deficient mice fail to develop Th2 $CD4^+$ cells [109, 110]. However, a small $CD4^+$ population with similar innate characteristics, as the $CD8^+$ T-cells in Itk-deficiency, has been shown in Itk-deficient mice [111]. Another cell type affected in Itk-defective mice is the NKT cell. The mice show a block of NKT cell maturation and reduced peripheral survival of NKT cells [112].

The different innate cell types described previously are known to be selected by non-classical MHC class Ib molecules on hematopoietic cells in the thymus. Itk-deficient

mice show a similar selection. However, a substantial portion of the altered $CD8^+$ T-cells in Itk-deficiency still requires class Ia for their selection [101]. Unlike the innate $CD8\alpha\alpha^+$ T-cells, the Itk-deficient $CD8^+$ T-cells express $CD8\alpha\beta$ instead of $CD8\alpha\alpha$ [100, 101, 113]. The characteristics of Itk-defective $CD8^+$ T-cells and the Wt innate cells do not exactly match.

Many Itk- and Itk-/Rlk-defective $CD8^+$ thymocytes are HSA^{lo}, which is a sign of a more mature phenotype. It was suggested that these $CD8^+$ cells might be mature peripheral cells that had migrated back into the thymus. However, studies have demonstrated that these innate-like memory T-cells develop in the thymus. [100, 101].

IL-15 is important for the development of innate $CD8^+$ T-cells deficient in Itk [100, 102]. In IL-15-deficient mice reduced numbers of $CD8^+$ T-cells with innate phenotype are seen. Mice with deficiencies in both IL-15 and Itk showed virtually no $CD8^+$ T-cells in peripheral tissues [100, 102]. Also signaling via the cell surface receptor signaling lymphocytic activation molecule (SLAM) has been implicated in the development and survival of innate T-cells such as NKT cells [114]. SLAM-associated protein (SAP) is an adaptor protein encoded by the gene SH2 domain protein 1A (SH2D1A). It is expressed by T-cells, NK-cells, NKT-cells, eosinophils, platelets and some B-cell populations. Mutations in the gene cause a severe primary immuno-deficiency disease known as X-linked lymphoproliferative syndrome (XLP) in humans. The symptoms are fatal Epstein-Barr-Virus (EBV)-induced infectious mononucleosis, subsequent hypogammaglobulinemia and increased risk of lymphomas [115]. In mice, SAP-deficiency can prevent the development of innate $CD8^+$ T-cells in Itk-deficient mice [105].

No human disease has ever been associated with mutations in Itk, until just recently when two girls were found to have a homozygous missense mutation in the SH2 domain of Itk causing fatal EBV-associated lymphoproliferative disease clinically resembling XLP. The mutation caused protein destabilization, lack of Itk in lymph node biopsies and absence of NKT cells. Also, increased level of the transcription factor eomesodermin was found in both girls [116].

1.3.3 Tec-deficiency

Tec is expressed in both B- and T-cells and it is activated via both BCR and TCR [117, 118]. The Tec-deficient mice develop normally and have no major phenotypic alterations of the immune system. They are more of a Wt mouse [74]. Similar expression of IgM and IgD was seen in Tec-deficient and Wt peripheral B-cells [74]. The T-cell development also appeared normal. Tec has been shown to be involved in the signaling mediated by CD28 co-stimulation [118]. However, experiments show equal responses to anti-CD28 for both Tec-deficient and Wt T-cells. Due to that no specific alteration in phenotype was seen in Tec-deficiency, another Tec kinase could potentially compensate for the loss of Tec. Ellmeier *et al.* created a Tec/Btk double-deficient mouse to find out if Btk has any compensatory role in B-cells. They could show that Btk compensates for Tec in Tec-deficient B-cells.

1.3.4 Itk activation

Itk is required for TCR-induced proliferation and IL-2 production [106, 108, 119]. Upon stimulation of the TCR (e.g. by anti-CD3), Itk is activated by the Src kinase Lck. Lck will phosphorylate the ITAMs of the intracellular tail of the CD3 chain. This will recruit ζ chain-associated protein kinase of 70 kDa (ZAP-70). Lck and ZAP-70 will together phosphorylate LAT and SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). They will form a signalosome that will recruit other signaling molecules such as Itk and PLCγ1. Itk phosphorylates PLCγ1 resulting in hydrolysis of PIP_2 into IP_3 and DAG. IP_3 will then bind to receptors in the endoplasmic reticulum releasing Ca^{2+} from the intracellular stores. This will then activate calmodulin and the Ca^{2+}-sensitive phosphatase Calcineurin, which leads to downstream activation of transcription factors such as NFAT [120]. This pathway is completed by the transcription of IL-2. DAG will activate mitogen-associated protein kinase (MAPK) pathways that will lead to activation of the Ras-Raf-ERK pathway and AP-1 acting on transcription.

For optimal activation of T-cells two signals are required. The first is from the TCR and the second from a co-stimulatory receptor such as CD28. CD28 is activated through its ligands B7-1 and B7-2 [121]. CD28 activates the Ser/Thr kinase protein kinase B (PKB, also known as Akt), which is an important factor in the PI3K pathway. Co-stimulation of T-cells via CD28 results in up-regulation of IL-2 mRNA levels and here

Akt phosphorylates S^{647} on NF90, which is an AU-rich element (ARE)-binding protein, resulting in that NF90 binds to 3´untranslated regions of IL-2 mRNA making it more stable by slowing down its degradation [122]. This is in contrast to the stimulation with CD3 only, where usually the signals go via PLCγ as described above. The question whether CD28-stimulation is dependent or independent on Itk has been discussed in two papers. One shows that Itk is a negative regulator of CD28 signaling [123] and the other declares that Itk is not essential for CD28 signaling in naïve $CD4^+$ T cells [124].

1.4 HIGH-THROUGHPUT TECHNOLOGIES NOW AND THEN

Microarrays have since the middle 1990's grown enormously and today the technique is routinely used in many laboratories. The first paper describing the use of microarrays was published in 1995. It describes differentially expressed genes in the plant *Arabidopsis thaliana* and complementary DNA (cDNA) arrays were used [125]. cDNA arrays are usually spotted in full length onto glass slides and are typical "in-house" printed microarrays that can be produced in the own lab. The probes are either Polymerase Chain Reaction (PCR)-amplified cDNA clones or expressed sequence tags (ESTs) [126]. Agilent microarrays contain 60 nucleotides (nt) long oligo probes [127]. The precise probe length has been tested to give highest specificity and sensitivity compared to short oligos [128]. The principle of cDNA arrays is that two target oligos with different fluorophores, such as Cy3 and Cy5, are mixed and hybridized together on a single microarray. The targets will bind to the probes in amounts reflecting their concentrations and hence abundance in each of the two analyzed samples. For each probe, a ratio between the signal intensities for the two targets is calculated [129].

Another type of array is the oligonucleotide arrays, which are made of short sequences of oligonucleotides designed to be specific for a gene. 25-mer and 60-mer long oligonucleotides have been used. Affymetrix is one of the biggest companies manufacturing short oligonucleotide arrays. Its technology will be described in more detail below.

Nowadays, splicing sensitive microarrays are becoming more and more popular. Those arrays have probes targeted to individual exons or exon-junctions and are able to detect splice isoforms. Genome-wide expression arrays have also been used more and more lately. Those arrays are called tiling arrays and covers the whole genome of an

organism, both coding and non-coding regions. They are normally used in chromatin immunoprecipitation (ChIP) experiments, where binding sites of proteins are mapped. Combining the two, results in a ChIP-on-chip that can identify binding sites of transcription factors across the genome. The method has been used for several organisms and applications, such as DNA methylation studies [130], new targets promoting prostate cancer growth [131], whole-genome studies in plants [132] and studies of Drosophila embryo development [133] among others. The first tiling array was covering human chromosomes 21 and 22 with probes spaced on average every 35 base pairs (bp) along the chromosomes [134]. The study was done to map the transcriptionally active areas in chromosomes 21 and 22 and the outcome was that a much larger proportion of the genome is transcribed and transported as cytoplasmic poly $(A)^+$ RNA than previously measured [134]. In another paper evaluating the same tiling arrays, novel transcripts were found to be expressed further supporting that the human genome is not fully understood yet and additional re-evaluations of the number of human genes must be done [135].

There have been studies criticizing the reliability of microarray data due to the inability to replicate the differentially expressed gene lists across similar experiments [136-138]. After reanalyses of one microarray study, the cross-platform concordance was improved after changing the method for classifying differentially expressed genes [139]. The MicroArray Quality Control (MAQC) project was started to evaluate the problems with reliability of the microarray technology. In that project, gene expression levels from two distinct RNA samples in four pools were analyzed on seven microarray platforms resulting in both inter- and intraplatform reproducibility [140, 141]. Other studies have also shown high consistency between different platforms [142, 143].

For more than ten years microarrays have been used in different applications, e.g. in cancer research looking for diagnostic markers related to the disease [144-147], drug response [148-150], toxicology [151, 152] and stress response studies [153]. So what has the future to offer?

The very new trend is called next generation sequencing [154]. Here the instruments are capable of producing millions of DNA sequence reads in a single run. A single short sequence read is determined for millions of nucleic acids from a biological sample. Only 25-35 bp of sequence allows you to bioinformatically identify the

location of each fragment in a reference genome. By the use of the so called Seq-based method you avoid some of the problems microarrays have, e.g. synthesis of millions of probes, cross-hybridizations of probes and difficulties with interpreting the hybridization signals. This technology will provide genome-wide sequence readouts in a number of different applications ranging from chromatin immunoprecipitation, mutation mapping and polymorphism discovery to noncoding RNA discovery. High-throughput sequencing and microarrays will probably complement each other more and more in the near future. There are already papers published that have compared data with the two techniques resulting in a more complete transcriptomic view when both were used [155, 156]. The technology is quite new and the cost and analysis of the enormous amounts of generated data are issues still needed to be worked with.

1.4.1 Affymetrix GeneChip® arrays

Affymetrix GeneChip® technology was invented during the late 1980's. The earliest descriptions of gene expression profiling using the Affymetrix GeneChip® platform was in 1996 [157]. The arrays consist of probe sequences of oligonucleotides. The sequences from which these probe sets are derived are selected from GenBank®, dbEST, and RefSeq data bases. The oligonucleotides are synthesized onto quartz wafers [158], which are treated with silane reagent intended for making the wafer surface susceptible for attaching so called linker molecules with photolabile-protecting groups. The surface can after that be activated by light. A photolithographic mask with windows that either block or permit the transmission of UV-light is then applied. This is called the photolithographic process and it will deprotect hydroxyl groups on the linker molecules and by that nucleotides are able to couple to them [159]. The deprotection and coupling procedure is repeated until the probe sequences are 25-mers long.

Affymetrix has three strategies for probe design (Figure 4). The in vitro transcription (IVT) expression probes are the most common type for whole transcriptome analysis. Affymetrix has probes for many different species, all from Arabidopsis to zebra fish. The probes are designed in the 3'-end of a gene in the region of 600 bases from the polyadenylation site. Eleven probe-pairs are usually designed for each gene on the array. Each probe-pair consists of a perfect match (PM) probe and a mismatch (MM) probe, containing the same PM sequence except for one nucleotide change at the 13th

position in the 25-mer long oligonucleotide. The 11 probe-pairs are usually called probe-set.

The exon array probes are designed to be four for each probe selection region (PSR), which is for each exon [160]. Many genes have more than a hundred probes on the exon array. Tiling arrays cover the whole genome, both coding and non-coding regions. The probes are 25-mers and tiled at an average resolution of 35 bp as measured from the central part of adjacent oligos, resulting in a 10 bp gap between them [161]. See Figure 4 for a schematic view of the three probe designs.

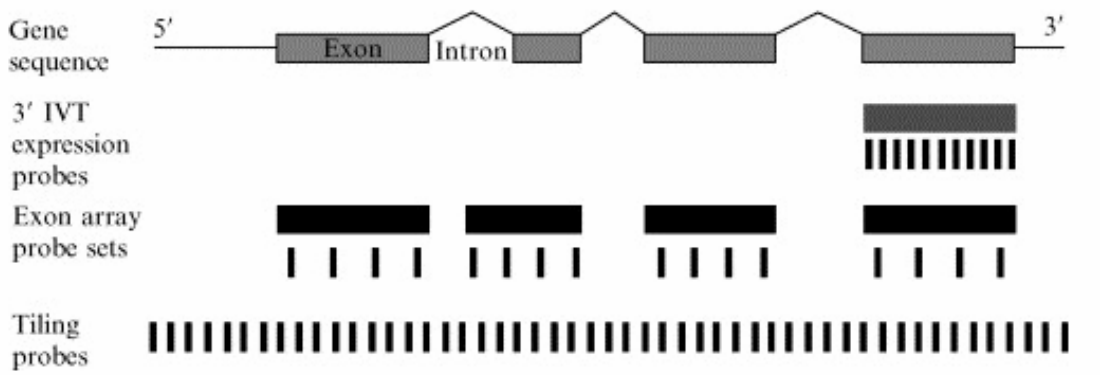

Figure 4. The three array design approaches from Affymetrix. The figure was obtained from [159].

The target preparation starts with isolating total RNA or DNA. RNA is reversed transcribed into cDNA by the use of a primer that includes a poly (T) tail and a T7 polymerase-binding site. Both first strand and second strand cDNA synthesis are performed. The double-stranded cDNA is then used in IVT where biotinylated nucleotides are incorporated into the target, resulting in a biotin-labeled cRNA sample. In cases of very small amounts of starting material (100 ng or even less) two rounds of IVT amplification is needed. The cRNA sample is then hybridized onto the microarray, which is stained with phycoerythrin-conjugated streptavidin prior to scanning (Figure 5). If using DNA as starting material, it is first purified and fragmented by DNase I digestion. The fragmented DNA is then labeled with terminal deoxynucleotidyl

transferase (TdT) and a biotinylated nucleotide analogue. After that the DNA can be hybridized onto an array.

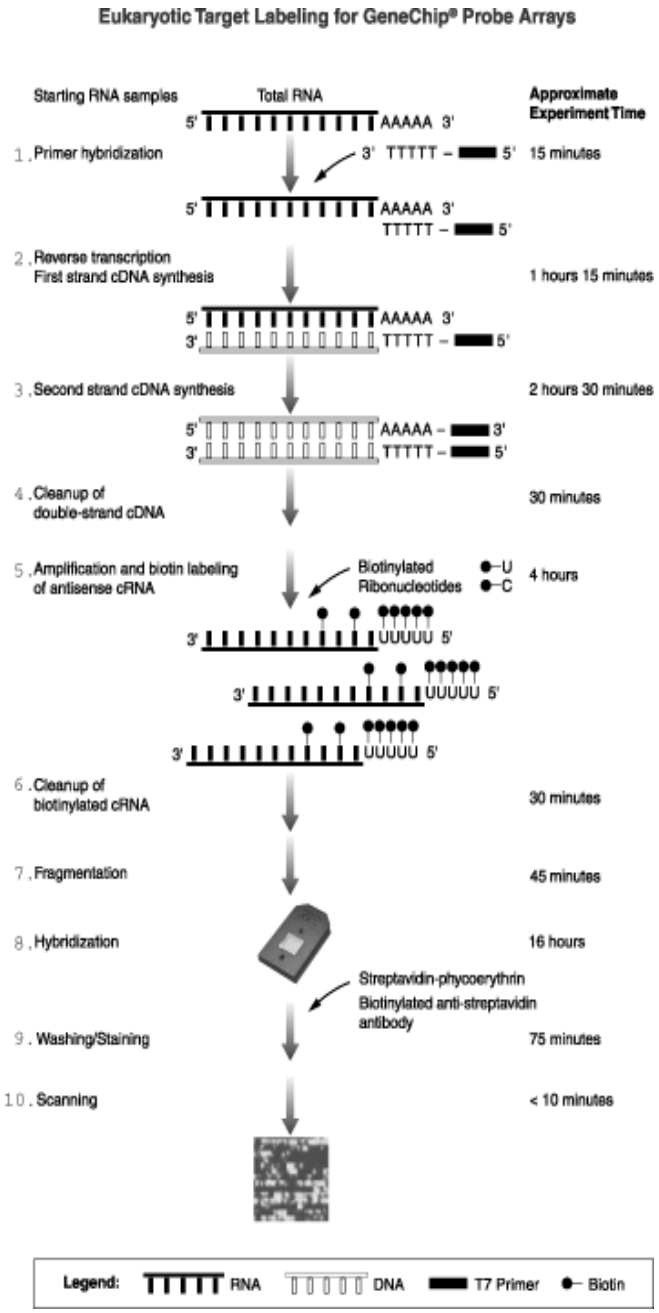

Figure 5. The different steps of preparing samples for an Affymetrix GeneChip® microarray experiment [www.affymetrix.com].

1.4.2 Experimental design

A good experimental design is the first thing one has to consider when setting up a microarray experiment. To be able to detect differentially expressed genes with a high level of confidence, one has to think about the number of replicates. This is done to control for both biological and technical variations. It has been a lot of debate about how many replicates that is sufficient. It is highly recommended to use at least three replicates [162, 163]. The situation is a bit different when you have limited amounts of sample. Pooling samples is a way to solve that issue and by that the effects of biological variation can be minimized [164, 165]. An amplification strategy (two rounds of IVT) is another way to deal with the problem with limited amounts of sample.

1.4.3 Image and data analysis

After the scanning process, the fluorescence signals are converted into electronic signals and then further into numerical values called fluorescent intensities. They are stored as pixel values in the image data file (.dat file). An additional step translates the .dat file into a .CEL file that is used by different analysis software to detect differentially expressed transcripts.

The analysis of microarray data includes many different steps. The first step after retrieving the raw data is normalization and background subtraction. This is done to compare one microarray to another.

1.4.3.1 Normalization

Array experimental conditions such as sample preparation and fluorescence labelling can strongly affect the microarray hybridization intensities. Normalization aims to remove (or minimize) this technical variation, so that we in the end only have data that differ due to the biological variation in the samples. Normalization can be applied within an array or between arrays. Some normalization methods perform a background correction by adjusting the PM probes by effectively subtracting the signal found on the MM probes, e.g. MAS5.0/Affymetrix GeneChip® Operating Software (GCOS) [166, 167]. Other methods compute background-corrected PM intensities for each PM cell on every array and don't take into account the MM probe intensities, e.g. RMA (Robust

Multi-array Average) [168] and the PM-only model-based expression strategy in dChip (DNA-Chip Analyzer) [169].

In most of our studies we have chosen to use the background-correction and normalization method in dChip. The normalization step in dChip is done on all arrays at the same time using, by default, an array with median overall intensity as baseline, against which other arrays are normalized. dChip uses a rank invariant set of normalization and model-based expression with PM-MM or PM-only for background subtraction. "Rank-invariant" genes are those whose expression values show a consistent order relative to other genes in the population [170, 171]. The above mentioned normalization methods have been compared in a number of studies [172-175] as well as further developed, such as GCRMA, which is a modified RMA method that takes into account the GC-content of the probes in the background adjustment. There is no simple answer to what method to choose, one has to try different ones.

1.4.3.2 Data analysis

After normalization the data can be further analyzed. Usually the aim is to find the amount of differentially expressed genes in a data set, e.g. finding the genes affected by a treatment, or finding marker genes that discriminate diseased from healthy subjects. Different methods to find patterns in gene expression data are available.

Some kind of filtering is usually good to start the analysis with. Filtering can be done to remove genes showing e.g. low variation over a whole data set or are not expressed at all. The usual approach to find differentially expressed genes is to apply a comparison criterion on the data, such as a fold difference where genes changing at least a specific fold between two conditions are selected. Also measuring the statistical significance of a finding is very important due to that the fold-change cut-offs do not take variability into account. Classical statistical tests are the Student's t test and analysis of variance (ANOVA). The t test tests the null hypothesis of equal mean expression levels in two samples. The t test is used when studying one categorical factor (e.g. stimulation) and ANOVA on multiple categorical factors (e.g. stimulation and time). There has been debate about using the normal t test in microarray data since it causes multiplicity problems due to testing of thousands of hypotheses. The most widely used approaches for statistical filtering currently involve some type of permutation or re-sampling analysis to produce a false-discovery rate (FDR) calculation to identify genes whose

expression change is likely to not have occurred by chance alone. Since tens of thousands of genes are compared, many genes can be false positives. There are several statistical methods taking this into account by doing multiple-comparison adjusted p-values, FDR calculations and resampling-based multiple tests [176-178]. The R programming language is widely used for statistical software development and data analysis of microarrays. Bioconductor, which is a genomic data analysis software, is based on the R programming language and has many tools for microarray data analysis.

Other ways to analyze microarray data are by grouping the genes in different clusters depending on some common variable such as function. Genes in the same cluster are more similar to each other than genes from different clusters. The similarity is often assessed by some kind of distance measure. This kind of analysis has been successfully used in different microarray studies [179, 180]. There is a variety of different cluster analysis types; hierarchical clustering, self-organizing maps (SOM), k-means clustering and principal component analysis (PCA) are only some of the types available [181-183]. The gene annotations are very important for the comprehension why some genes cluster together. The Gene Ontology (GO) Consortium (www.geneontology.org) provides three controlled vocabularies to annotate biological knowledge. The three terms are biological process, cellular component and molecular function. Statistical tools have been developed to find over- or under-represented GO terms within lists of genes [184].

1.4.3.3 Validation of the data

After retrieving a list of statistically significantly changed genes, one has to verify the findings with some other method. Usual methods are quantitative Reverse Transcriptase (RT)-PCR, Western blot or ELISA. The methods are described in more detail in the Materials and methods section.

2 AIMS

General aim: To investigate the gene expression profiles of the mouse models having defects in the different Tec family kinases in order to study common patterns but also to learn about their transcriptomic differences.

Specific aims:

Paper I

- To study the gene expression profile of Btk-defective mouse splenic B-cells compared to Wt cells

- To reveal differences between Btk KO and Xid mouse at the transcriptional level

Paper II

- To investigate the transcriptional fingerprints of Btk-deficiency at the T1 stage of B-cell development

Paper III

- To characterize the transcriptome of Itk-deficient $CD3^+$, $CD4^+$ and $CD8^+$ T-cells

- To study if Itk-deficiency mimics calcineurin inhibition by treating Wt T-cells with cyclosporin A

- To identify common transcripts regulated in Itk-deficient T-cells and Btk-deficient B-cells

Paper IV

- To compare the gene expression profiles of $CD3^+$ T-cells from Tec-, Itk- and Itk/Tec-deficient mice and define the transcriptomes of Tec- and Itk/Tec-deficient mice

3 MATERIALS AND METHODS

3.1 MOUSE MODELS

Xid/CBA mice were obtained from Charles River Laboratories, Sweden, Btk KO/CBA mice were created by back-crossing Btk KO/SW129 mice on CBA background strain for nine generations and normal CBA mice were used as controls (**Papers I and II**).

Itk- [106], Tec- and Itk/Tec-deficient mice were back-crossed on C57BL/6 mice (obtained from Wilfried Ellmeier, Institute of Immunology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, Austria). Wt C57BL/6 mice were used as controls.

The mice were housed in isolators with sterile plastic boxes provided with high efficiency air filters that keep the mice free from infections. This is important when working with immunodeficient animals, since infections could influence the phenotype and result in incorrect interpretations.

3.2 B- AND T-CELL SEPARATIONS

For **Paper I**, splenic B-lymphocytes were stained with anti-B220 antibody and enriched using a high-gradient magnetic separation column (Miltenyi Biotec). The purity of the cells was checked by FACS analysis with PE-conjugated rat anti-mouse-CD19 antibodies and resulted in a purity of >92%. For **Paper II**, the B-lymphocytes were isolated from spleen of 1-week-old mice and cells were sorted by FACS Diva in the same way as in **Paper I**.

In **Paper III and IV**, $CD3^+$, $CD4^+$ and $CD8^+$ T-cells were isolated by negative depletion using the antibodies bio-anti-CD11b (M1/70) and bio-anti-CD11c (HL3) (BD Pharmingen) and bio-anti-CD45R (B220), bio-anti-Ly-6G (RB6-8C5), bio-anti-erythroid cells (Ter119) and bio-anti-NK1.1 (PK136) (Caltag). When $CD4^+$ and $CD8^+$ T-cells were isolated, bio-anti-CD8α (5H10) and bio-anti-CD4 (CT-CD4) antibodies (Caltag) were used, respectively. Streptavidin beads (BD Pharmingen) were used to bind to the biotin-conjugated antibodies. High-gradient magnetic separation columns

were used for the negative depletion (Miltenyi Biotec). The purity of the cells was >90% $CD3^+$, >96% $CD4^+$ and >90% $CD8^+$ T-cells.

3.3 CELL STIMULATIONS

The splenic B-cells in **Paper I** were stimulated with anti-IgM (40μg/ml), LPS (20μg/ml) or PMA + ionomycin (1μg/ml of each) for 2 and 6h prior to quantitative RT-PCR analysis. In **Paper III** the $CD3^+$ T-cells from Wt and $Itk^{-/-}$ mice were unstimulated as well as stimulated with anti-CD3 (1μg/ml) ± anti-CD28 (3μg/ml) for 24h. CsA-treatment (1μg/ml) was done on Wt $CD3^+$ T-cells with anti-CD3 (1μg/ml) ± anti-CD28 (3μg/ml) for 24h as well. The $CD4^+$ and $CD8^+$ T-cells were unstimulated as well as stimulated with anti-CD3 only for 24h. In **Paper IV** the T-cells were unstimulated as well as stimulated with anti-CD3 (1μg/ml) for 24h and anti-CD3 (1μg/ml) + anti-CD28 (3μg/ml) for 12, 24 and 42h.

3.4 RNA ISOLATION

Total RNA isolations were performed using TRIzol (Gibco BRL) (**Paper I**) and RNeasy Mini kit (Qiagen, Valencia, CA, USA) (**Papers I-IV**). The RNA amount and the quality were measured with a NanoDrop spectrophotometer (Wilmington, USA) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). In Figure 6 is an example of an RNA sample measured with the Agilent Bioanalyzer.

For **Paper I** we used 10 μg of total RNA for hybridization on the Affymetrix GeneChip®. In **Papers II-IV**, 100 ng of total RNA was used and here the RNA was amplified in Affymetrix Small Sample Protocol with two rounds of IVT.

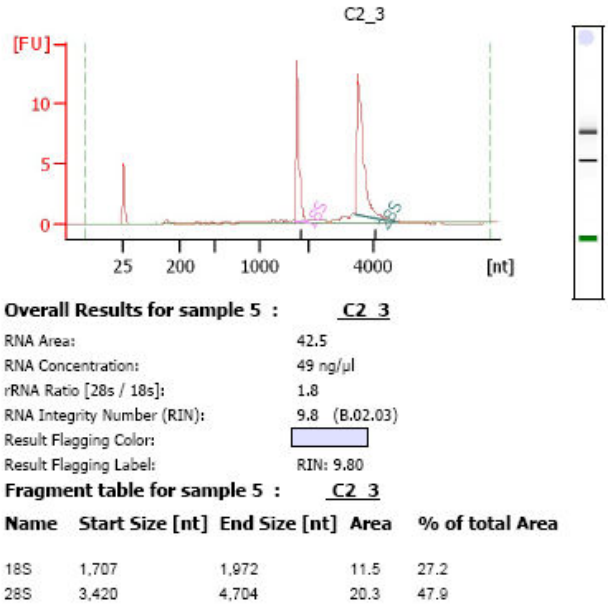

Figure 6. RNA concentration and quality measured with Agilent Bioanalyzer 2100. The RNA sample is from mouse Wt $CD3^+$ T-cells stimulated with anti-CD3 for 24h.

3.5 AFFYMETRIX MICROARRAYS

In **Paper I** we used Affymetrix GeneChip® oligonucleotide mouse array U74Av2 containing more than 12,000 genes and ESTs. Duplicate samples of CBA, Btk KO and Xid and a total of six arrays were used. In **Papers II-IV** we used Affymetrix MOE430 2.0 GeneChip® with 45,000 probe-sets covering the mouse genome. In **Paper II**, 10 arrays were run; four arrays for the CBA and Xid mouse groups and three arrays for the Btk KO strain. In **Paper III**, a total of 37 arrays were run. In **Paper IV**, a total of 55 arrays were run (some overlap with **Paper III**).

The cRNA synthesis and hybridizations were performed in the Affymetrix core facility at Novum, BEA, Bioinformatics and Expression Analysis, Karolinska Institutet, Huddinge, Sweden.

3.6 DATA AND STATISTICAL ANALYSIS

The data in **Paper I** was analyzed using Affymetrix Microarray Suite version 4.0 and DMT version 3.0. A scaling factor of 100 was used for normalization. Initial analysis of **Paper II** was performed using the Affymetrix GeneChip® Operating Software

(GCOS) version 1.2 and further analysis was done with dChip (found at http://www.hsph.harvard.edu/~cli/complab/dchip/). In **Paper III and IV**, all the analyses were done in dChip. Comparative analyses have been done in all the papers and all with a 2-fold-change criterion.

In Paper I, only a 2-fold-change analysis was applied due to few replicates per group. A fold difference of >2 and <-2 in Xid or Btk KO compared to Wt was considered significant. Also genes differing >2-fold between Btk KO and Xid samples were considered further.

In **Paper II**, the dChip software was used to study differentially expressed genes. Invariant set normalization was done. The following criteria were used in the comparison analysis between Btk-defective (Btk KO and Xid) and controls: fold-change was set to more than 2 and less than -2. The statistical significance between the whole splenic B-cell data and T1 B-lymphocytes were calculated using the HYPGEOMDIST function in Excel. Hierarchical clustering was performed on filtered data. In **Paper II** we also did a Marker analysis by the use of GeneCluster2 [185] in order to identify genes correlated with a particular class distinction, Btk-defective (Btk KO and Xid) versus control (CBA). 50 markers were chosen for the correlation of each class. The 50 probe-sets being most similar within the group and the 50 being most dissimilar between groups were selected.

In **Papers III and IV** we used dChip for invariant set normalization and model-based expression calculations according to the PM-only model. A fold-change of ≥ 2 between groups was used. The Student's t test in Excel as well as in dChip was used for hypothesis testing. $p<0.05$ was set as threshold for statistically significant genes. In **Paper IV** a hierarchical clustering was performed on a filtered list.

3.7 QUANTITATIVE RT-PCR

Total RNA was reverse-transcribed into cDNA with AMV Reverse Transcriptase using random hexamer primers (Roche Applied Science,IN, USA). Pre-developed TaqMan® Gene Expression Assays (Applied Biosystems) containing probe and primers for genes evaluated in the microarrays were used with the ABI Prism® 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). This technique was used in **Paper I and III**.

The TaqMan® probes have a reporter fluorophore in its 5´ end and a quencher in its 3´end. This assay is based on the fluorescence resonance energy transfer (FRET), which means that when the probe is intact the fluorescence emission of the reporter is absorbed by the quencher. As soon as the probe has annealed to a target sequence and the Taq DNA polymerase cleaves the probe, in the extension phase of the PCR reaction, the reporter and quencher will be separated from each other and fluorescence can be detected and measured [186]. Quantification can either be relative to an external standard curve or to one or more co-amplified internal control mRNAs. Quantification relative to internal control mRNAs compares the *C*t (threshold cycle) values from target RNAs to those of one or more internal reference genes (housekeeping genes) and results are expressed as ratios of the target-specific signal to the internal reference.

Relative quantification was done according to the comparative Ct ($2^{-\Delta\Delta Ct}$) method. 18S ribosomal RNA (18S rRNA) was used as endogenous control.

3.8 WESTERN BLOT

Western blot is a technique to identify and locate proteins with the help of specific antibodies. The proteins are analyzed from cells that have been lysed with different detergents. The proteins in the lysate are separated, by molecular mass, on SDS-PAGE (polyacrylamide) gels using electrophoresis. After that the proteins are blotted onto nitrocellulose membranes using an electrical field. The transferred proteins are bound to the surface of the membrane, providing access for reaction with antibodies. The membrane is first blocked to prevent unspecific antigen interactions. Then the membrane is probed with a primary antibody, which recognizes specific antigen. The antibody-antigen complexes are identified with horseradish peroxidase (HRP) enzyme conjugated to the secondary anti-IgG antibody (species specific). The HRP-conjugated antibody can easily be detected with a chemiluminescence substrate (ECL), producing luminescence (photon) signal that can be detected by a phosphoimager instrument or with photography film. This technique was used in **Paper I and IV** to confirm microarray findings. The proteins in **Paper IV** were resolved on 4-12% gradient NuPage gels (Invitrogen) and transferred to nitrocellulose membranes using the dry blotting system iBlot (Invitrogen).

3.9 FLOW CYTOMETRY

Cells can be counted or sorted out from a pool of cells by the use of antibodies that bind to their cell surface. The antibodies are conjugated with fluorescent dyes, which react on light of a specific wave length. The cells labelled with the antibody + dye are run through the FACS machine in a stream of fluid and at the same time a beam of light is directed against the stream. Detectors in the flow cytometer pick up the light that is emitted by the fluorescent chemicals.

In **Paper I** we confirmed the down-regulation of CD9 expression in Xid mice at protein level by flow cytometry. Also, the purity of the $CD19^+$ splenic B-cells was checked. In **Paper II**, $CD19^+$ (PE-conjugated rat anti-mouse CD19 antibodies) T1 cells were sorted in a FACS Vantage Diva Cell Sorter, Beckton Dickinson, and the purity was checked with FACS as well. In **Paper III and IV**, the purity of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells was evaluated with flow cytometry.

3.10 ELISA

ELISA is short for Enzyme-linked immunosorbent assay and is used to quantitate and detect an antigen or an antibody in a solution. An antigen is usually bound to the bottom of a well in a microtiter plate and a solution of antibodies is added to the plate. A secondary antibody, conjugated with an enzyme or fluorescence, will bind to the primary antibody and through an enzyme-reaction or adding light with a specific wave length, the amount of antigen/antibody in the solution can be measured. This method is a useful tool for determining serum antibody concentrations.

In **Paper I** we coated ELISA plates with rabbit anti-mouse-Ig (Dako-Cytomation Denmark A/S, Glostrup, Denmark). Serum samples from mEH-deficient mice as well as control mice were added to the plate and alkaline-phosphatase-conjugated rabbit anti-mouse-IgG3 (ICN/Cappel, Aurora, OH, USA) or goat anti-mouse-IgM (Jackson ImmunoResearch laboratories Inc., West Grove, PA, USA) antibodies were added. The plates were measured in a multichannel spectrophotometer (Labsystems Multiscan Plus, Finland).

3.11 CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) is a technique used for finding proteins associated with specific regions of the genome. This is done by the use of specific antibodies that recognize a protein or a modification of a protein. The DNA-binding proteins, such as transcription factors, are first cross-linked to the DNA by the use of formaldehyde. After cross-linking, the DNA is broken down into smaller fragments (on average 500 bp) by sonication. The protein-DNA complexes are then immunoprecipitated by an antibody against a protein of interest. The cross-linking is reversed and DNA is purified. Then the DNA is used in PCR amplification using primers that are specific to a region in the DNA [187]. This is a way to study transcription factor binding sites in promoter regions.

This technique was used in **Paper III** where we could show binding of NFATc1 (sc-1149-R, Santa Cruz Biotechnology, Inc.) to the promoter regions of *IL7R, Bub1, Ctla2a, Ctla2b* and *Slfn1* genes.

4 ETHICS

All studies were performed in accordance with approved ethical permissions. Dnr: S4-99 (**Paper I**), Dnr: S182-01 (**Paper I**), Dnr: S94-03 (**Paper II**), GZ 66.009/265-BrGT/2004 from Austria (**Papers III and IV**).

5 RESULTS AND DISCUSSION

By studying Btk-deficiency in B-cells and Itk-, Tec-, and Itk/Tec-deficiency in T-cells with the help of gene expression profiling, we have collected a large amount of data. We started out with the mouse U74Av2 chip covering >12,000 genes and ESTs, and proceeded with newer and more annotated GeneChip® arrays (MOE430 2.0) in the later studies.

5.1 PAPER I

Gene expression profile of B cells from Xid mice and Btk knockout mice
Lindvall JM, **Blomberg KEM**, Berglöf A, Yang Q, Smith CIE and Islam TC.
Eur J Immunol. 2004 Jul;34(7):1981-91.

In this paper we sought to determine the resemblances and differences in gene expression profile of Btk-defective mice in comparison to healthy mice on the same background (CBA). Total splenic $CD19^+$ B-cells from CBA (control), Xid and Btk KO mice were isolated in duplicate. The Xid phenotype is caused by a missense mutation (R28C) in the PH-domain. Btk KO mice lack detectable levels of Btk protein and have an Xid-like phenotype [66, 67, 188]. The expression signatures of these mice were analyzed by the use of Affymetrix mouse U74Av2 GeneChip® arrays, covering more than 12,000 mouse genes and ESTs.

A gene-average of 49% was expressed in all samples and a total of 4515 probe-sets (36% of all genes on the chip) were expressed in duplicate experiments. When looking for differentially expressed transcripts, the fold-change criterion >2 or <-2 was used. Of the 4515 probe-sets, 38 genes were differentially expressed between Btk-defective (overlapping in both Xid and Btk KO) and control mice. Among the 38 genes, a factor called microsomal epoxide hydrolase (mEH/Ephx1) was found largely down-regulated (-27-fold). mEH was shown to have a role in metabolizing the epileptic drug phenytoin, which is a drug that can cause IgA-deficiency. Deficient detoxification of endogenous metabolites could potentially impair B-cell development. We excluded a functional relationship between mEH and Btk-deficiency by showing that mEH-deficient mice did not have low IgM and IgG3 levels and B-cell numbers. We could confirm the down-regulation on protein level using immunoblots.

Other genes that were down-regulated were CD9 antigen (-5.45-fold) and the transcription factors Ikaros (-2.70-fold) and NFATc1 (-3.80-fold). The most up-regulated genes were ionized Ca^{2+} binding adapter molecule 1 (Iba1) (7.47-fold) and insulin-like growth factor 1 (Igf1) (6.50-fold). Iba1 was found to have a binding site for the transcription factor PU.1 in its promoter. PU.1 is known to regulate Btk [189], but in our data it is not found. We can not find any mechanism underlying the up-regulation of Iba1.

To further investigate if there is any difference between an Xid mouse and a Btk KO mouse we compared them. Eight probe-sets, corresponding to 7 genes, were found to be differentially expressed between the two. All but one gene belonged to the 38-gene list with genes differing between Btk-defective and control mice. The new gene was interferon activated gene 202 and it was differently regulated in the two mice (3.0-fold in Btk KO and -3.5-fold in Xid).

We also conducted quantitative Real-time PCR on ten genes from the 38-gene list as well as five additional genes that were shown to be regulated in response to anti-IgM-stimulation in primary B-cells from "Btk-low mice" and normal mice in another study [190]. Splenic B-cells from CBA, Xid and Btk KO mice were stimulated with anti-IgM, LPS or PMA + ionomycin for 0, 2 and 6 hours. The 0-hour time-point correlated well with respect to the up- and down-regulated genes in the microarray data. After stimulation, seven out of the 15 genes showed a similar response pattern for all the three stimuli whereas the remaining eight had dissimilar response patterns. Anti-IgM and PMA + ionomycin stimulations showed more similar responses compared to the LPS stimulus.

5.2 PAPER II

Distinct gene expression signature in Btk-defective T1 B-cells
Lindvall JM, **Blomberg KEM**, Berglöf A and Smith CIE.
Biochem Biophys Res Commun 2006 Jul 28;346(2):461-9.

Recent studies have divided the mouse immature B-cells (HSA^{high}) into T1 and T2 cells [191]. T1 and T2 cells express distinct surface markers and are located in distinct anatomic locations. T1 cells give rise to T2 cells, whereas T2 cells can further

differentiate into mature follicular B-cells (HSA^{low}) [191]. The developmental block in Xid mice has been shown to be manifested at the T2 stage of B-cell development, thus it has only been shown with respect to surface markers [191-193].

Here we analyzed the possibility of an earlier developmental block, than previously reported for Btk-defective B-cells, by using Affymetrix MOE430 2.0 high-density oligonucleotide arrays with over 45,000 probe-sets covering the mouse genome. We collected splenic B-cells from 1-week-old control CBA (four replicates), Xid (four replicates) and Btk KO (triplicates) mice. In the spleens of 1-week-old mice all the B-cells are of T1 origin [191]. Extrapolating the 38 genes found in our first study (Paper I) on the T1 data-set, we found an overlap of 12 transcripts being statistically significant. The number of differentially expressed transcripts between Xid vs CBA and Btk KO vs CBA generated an overlapping list of 225 transcripts. The same fold-change criterion as in Paper I was used and the dChip comparative analysis was applied. Since Xid and Btk KO are so similar and there is no selective advantage in being Xid over Btk KO [39] we decided to combine the Xid and Btk KO mice into one Btk-defective group. Btk-defective samples were compared to controls and the result was a list of 147 transcripts (67 up- and 80 down-regulated). All the 147 transcripts were present in the 225-list.

A marker analysis was performed using GeneCluster2. Of the 45,101 probe-sets in the data, 3048 passed the variation filter for the Btk-defective vs CBA data-set. They were further used for marker analysis using the Signal-to-Noise ranking method. Among the best-correlated transcripts was mEH/Ephx1 showing up as a marker gene for Btk-deficiency. This gene was also found in the comparative analysis between Btk-defective vs control mice. Looking at the overlapping transcripts between the 38-gene list in Paper I and the 147-list the number was four, one of them being mEH/Ephx1. Interestingly, in the 147-list there is four times more transcripts than in the 38-list, and the situation for the arrays used is that the MOE430 2.0 arrays (45,000 probe-sets) have approximately four times more probe-sets than U74Av2 arrays (12,000 probe-sets) used in Paper I. This suggests that there was no skewing of the probes selected for this GeneChip with regard to genes differentially expressed.

The 147-list was used for reanalyzing the data obtained from whole splenic B-cells in Paper I. A Student's t-test was performed with the p-value threshold of $p<0.05$ for genes being significantly differentially expressed. Sixteen probe-sets had a p-value of less than 0.05 and were considered significantly changed between Btk-defective and control mice (three of them were found in the 38-list). The remaining 131 probe-sets were divided as follows: (1) 103 transcripts were not found on the U74Av2 GeneChip®. (2) The remaining 36 probe-sets were not statistically significant ($p>0.05$) between Btk-defective and control samples. This cross-analysis confirmed overlapping as well as new genes comparing whole splenic B-cells and T1 B-cells, supporting our hypothesis that the Btk defect is already manifested at the T1 stage.

5.3 PAPER III

Transcriptional signatures of Itk-deficient $CD3^+$, $CD4^+$ and $CD8^+$ T-cells
Blomberg KEM, Boucheron N, Lindvall JM, Yu L, Raberger J, Berglöf A, Ellmeier W and Smith CIE. Submitted

In this study we have characterized the transcriptome of Itk-deficient $CD3^+$, $CD4^+$ and $CD8^+$ T-cells. The challenge here has been to deal with the different T-cell populations in Wt and $Itk^{-/-}$ mice, where almost all the T-cells of Itk-deficient mice are of an innate-like phenotype [100, 101, 111]. Due to this we have sorted and enriched $CD4^+$ and $CD8^+$ populations of $CD3^+$ T-cells from both mouse strains. We started out comparing the total $CD3^+$ populations in unstimulated as well as anti-CD3/CD28- and anti-CD3-stimulated (both for 24h) Wt and Itk-defective T-cells. Comparing the two in each condition resulted in more differentially expressed transcripts in the untreated cells than in the treated. After co-stimulation the number of transcripts was decreased by approximately 50% compared to the anti-CD3-stimulated state. This suggests that the co-stimulatory pathway is less dependent of Itk.

We wanted as well to distinguish if the differences seen in Itk-deficiency compared to Wt were also seen at the $CD4^+$ and $CD8^+$ transcriptomic T-cell levels. Here we showed that more transcripts were differentially expressed in $CD8^+$ T-cells from $Itk^{-/-}$ compared to Wt than in $CD4^+$ T-cells. Interestingly, the gene Zbtb16, encoding the transcription factor promyelocytic leukemia zinc finger (PLZF), was found up-regulated in $CD4^+$ T-cells while down-regulated in $CD8^+$ T-cells from Itk-deficient mice. This factor was further confirmed to be important for development of $CD44^{hi}$ $CD4^+$ T-cells [194]. An

interesting finding in all the cell populations was the up-regulated levels of killer cell lectin-like receptors (Klrs) in Itk-deficiency. Eleven members were found in the $CD3^+$ T-cell pool and four were found in common with $CD4^+$ and $CD8^+$ populations. These receptors are usually expressed on NK-cells [195]. Three of the Klrs; Klrg1, Klra3 and Klra7, were confirmed with another method, namely quantitative RT-PCR. Granzyme M was found to be up-regulated in $CD8^+$ Itk-defective T-cells. This cytosolic protein has previously been shown to characterize NK-cells and cytotoxic T-cells with innate function [196, 197].

Treating cells with CsA will cause inhibition of calcineurin and by that the signaling via NFAT is defective. The Itk kinase is expressed upstream of NFAT and will as well have an effect on that pathway. We investigated which of the Itk-related changes was also calcineurin-dependent with or without stimulation (anti-CD3 or anti-CD3/CD28 for 24h). A large overlap between Itk-deficient samples and CsA-treated $CD3^+$ Wt samples was found, indicating that Itk-deficiency mimics the calcineurin inhibition. However, CsA had a much greater effect on transcriptional regulation, since it completely blocks calcineurin and further downstream signaling, compared to loss of Itk. 113 probe-sets were in common between the Itk-defective and CsA-treated cells independent of stimulation. Among them was Zbtb16 again, being both Itk- and calcineurin-dependent. IL-2 was also found to be in that group, confirming what is previously known [118, 198]. The smallest group of transcripts was the Itk-dependent and calcineurin-independent with 89 transcripts shared between the two stimulation conditions. Here, Klra5, Klra8 and Klre1 were found. The group with most differentially expressed transcripts was the calcineurin-dependent and Itk-independent one.

We also identified novel NFAT target genes by looking for possible NFAT-sites (GGAAA) in promoter regions of transcripts being both Itk- and calcineurin-dependent after anti-CD3-stimulation. Of 482 transcripts 24 were selected for bioinformatic analysis. The GGAAA sequence was searched for in the 500 bp region upstream of each of the transcript's transcriptional start site. 15/24 had one or more putative NFAT-sites in that region. We found *IL7R*, *Bub1*, *Ctla2a* and *Ctla2b* with 1 to 2 sites in that region, as well as the *Schlafen1* (*Slfn1*) gene, which had an NFAT-site upstream of the translation initiation site. ChIP experiments were performed to find out if NFAT bound to the promoter regions of the above mentioned genes. The experiments

resulted in binding of NFATc1 to all the five genes after anti-CD3-stimulation as well as to the IL-2 promoter region, as expected.

In Paper I we generated a list of 38 genes differentially expressed between Btk-defective and control mice. When comparing the 38-list with the list of differentially expressed transcripts in Itk-defective vs Wt mice we find an overlap of 18 genes. Sixteen of the 18 genes were similarly regulated, showing a highly significant co-variation. One has always assumed that Btk and Itk are each others counterpart in B- and T-cells, respectively. Though, thinking about their roles and what they actually accomplish, they are quite different. Itk is induced by the IL-2 cytokine, thereby the name, while Btk is not induced in the same way. We also have differences when it comes to human diseases, where we have mutations in Btk causing the B-cell defect XLA and the recent report describing mutations in Itk causing an XLP-like syndrome with defects in NKT cells [116]. Btk and Itk are not exactly each others counterparts; there are in fact fundamental differences between B- and T-cells over all.

5.4 PAPER IV

Tec and Itk regulate overlapping signaling pathways in T lymphocytes
Blomberg KEM, Boucheron N, Lindvall JM, Vargas L, Raberger J, Berglöf A, Ellmeier W and Smith CIE. Manuscript

The gene expression patterns from Tec-, Itk- and Itk/Tec-deficient $CD3^+$ T-cells were compared to Wt control $CD3^+$ T-cells in this whole-genome study. 55 arrays were run with at least duplicate samples in each mouse group and condition. Both untreated as well as anti-CD3/CD28- and anti-CD3-stimulated cells were analyzed at different time-points (12, 24 and 42h for co-stimulation and 24h for anti-CD3-stimulation). The number of differentially expressed genes in $Tec^{-/-}$ T-cells was small compared to Wt; the biggest difference was seen after 42h of anti-CD3/CD28-stimulation where 15 probe-sets differed (Table 1). These few alterations are in concordance with the previous finding that Tec-deficient mice develop normally and have no major phenotypic alterations in either B- or T-cells [74].

Table 1. Differentially expressed probe-sets in respective mouse group compared with corresponding Wt sample

	Samples compared to Wt	Number of probe-sets changed ≥2-fold and p<0.05
	$Tec^{-/-}$ unstimulated	14
anti-CD3/CD28	$Tec^{-/-}$ 12h	8
anti-CD3/CD28	$Tec^{-/-}$ 24h	8
anti-CD3/CD28	$Tec^{-/-}$ 42h	15
	$Tec^{-/-}$ anti-CD3 24h	11
	$Itk^{-/-}$ unstimulated	150
anti-CD3/CD28	$Itk^{-/-}$12h	450
anti-CD3/CD28	$Itk^{-/-}$ 24h	105
anti-CD3/CD28	$Itk^{-/-}$ 42h	149
	$Itk^{-/-}$ anti-CD3 24h	326
	$Itk^{-/-}/Tec^{-/-}$ unstimulated	143
anti-CD3/CD28	$Itk^{-/-}/Tec^{-/-}$12h	484
anti-CD3/CD28	$Itk^{-/-}/Tec^{-/-}$ 24h	183
anti-CD3/CD28	$Itk^{-/-}/Tec^{-/-}$ 42h	253
	$Itk^{-/-}/Tec^{-/-}$anti-CD3 24h	937

More transcripts were differentially expressed in $Itk^{-/-}$ and $Itk^{-/-}/Tec^{-/-}$ T-cells compared to Wt (Table 1). Rather equivalent amounts of transcripts were shown to be expressed in $Itk^{-/-}$ vs Wt and $Itk^{-/-}/Tec^{-/-}$ vs Wt T-cells (Table 1), but the biggest difference was seen after anti-CD3-stimulation for 24h (compare 326 with 937 probe-sets).

Bilic *et al.* summarized in a paper in 2007 five Bric-a-brac, tramtrack, broad complex domain-containing family of zinc finger proteins (BTB-ZF) that have a role in T-cell development and/or T-cell function; they were MAZR, Th-POK, Bcl6, Bcl6b and PLZP (Table 2). The BTB-ZF is a group of transcription factors, which has been shown to be involved in the cell fate and function of hematopoietic cells. The BTB domain is also called POZ domain (poxvirus zinc finger) and is an evolutionary conserved protein-protein interaction domain [199, 200]. These transcription factors bind DNA with their C_2H_2 zinc finger motifs resulting in chromatin modification, and localized transcriptional activation or repression [201, 202].

Some of the genes encoding those proteins were found in our T-cell data, as well as several others with similar nomenclature. One factor that should be included to the list of the five proteins is Zbtb16 encoding the protein PLZF. It was recently shown to be a regulator of $CD44^{hi}$ memory phenotype $CD4^{+}$ T-cells, the same population that is being enriched in Itk-deficient mice [194]. The other genes found in our data were Zbtb4, Zbtb20 (encoding DPZF) and Zbtb26. Further studies have been performed on the latter one, resulting in confirmed increased protein levels in Itk-defective $CD3^{+}$ T-cells (lymph node and spleen mixed) compared to control. The up-regulation was only seen in untreated samples, while no difference was seen after stimulation. A common trend seen with all the three new factors found in our data is that they are all up-regulated in $Itk^{-/-}$ or $Itk^{-/-}/Tec^{-/-}$ samples compared to Wt. More work has to be done to further decipher the roles of Zbtb4, Zbtb20 and Zbtb26 in T-cells.

Table 2. The different BTB-ZF factors in T-cells

Protein names in mouse	Gene name	Human homologs
MAZR	*Zfp278*	ZN278_HUMAN, PATZ
Th-POK, cKrox, Zfp67	*Zbtb7b*	ZBTB7B, hcKrox
Bcl6	*Bcl6*	BCL6, Znf51
Bcl6b, BAZF	*Bcl6b*	ZNF62, Bcl6b
PLZP, ROG TZFP	*Zbtb32*	ZBTB32, FAZF

6 CONCLUDING REMARKS

Microarrays have been a powerful tool for more than a decade. In this thesis the technology has been used in all papers resulting in an interpretation of the transcriptomic patterns of Btk-deficient B-cells and Tec-, Itk-, and Itk/Tec-deficient T-cells. Many questions have been answered but also many new ones have arisen and further experiments have to be done in order to solve them. Several transcription factors of potential interest have showed up in each of the studies, but more experiments have to be performed to unravel their specific roles. We now have data from different cell types and from different Tec family kinase mutants, therefore a more meta-analytic study can be conducted in order to decipher if there are more similarities between Btk and Itk. The findings in this thesis will hopefully contribute to some more understanding of the regulation of Tec family kinases and also to future challenges.

7 SAMMANFATTNING PÅ SVENSKA

Med några få undantag innehåller varje cell i vår kropp en komplett uppsättning kromosomer (46 st) och identiska gener. Endast en bråkdel av dessa gener är påslagna och det är detta uttryck man brukar prata om och som karakteriserar en viss celltyp. Genom att studera vilka gener som är aktiva och inaktiva i olika celltyper kan man förstå hur dessa celler fungerar normalt och hur de påverkas när olika gener inte fungerar ordentligt. Tidigare har man bara kunnat genomföra genetiska analyser av ett fåtal gener på en gång. Utvecklingen av DNA microarray-tekniken har nu gjort det möjligt att undersöka hur aktiva eller inaktiva tusentals gener är samtidigt.

När en gen aktiveras börjar det cellulära maskineriet att kopiera vissa delar av denna gen. Den resulterande produkten kallas budbärar-RNA (mRNA), som är kroppens mall för att skapa de livsnödvändiga proteinerna. Det mRNA som produceras av cellen motsvarar den del av DNAt den kopierats ifrån och RNAt kommer därför att "komplementärbinda" in till den ursprungliga delen av DNAt. Detta utnyttjar man i microarray-tekniken då man samlar in RNA från det man ska studera och märker in det med någon typ av fluorescerande färgämne. Det inmärkta RNAt sätts sedan till en microarray som består av miljontals oligonukleotider, vilka är delar av olika DNA molekyler (oftast DNA som motsvarar en hel organisms arvsmassa). Det RNA som finns i cellen kommer att binda in till oligonukleotiderna och i och med det märks de in med fluorescens. En scanner kommer sedan att läsa av microarrayen och beroende på hur många RNA-molekyler som bundit in till oligonukleotiderna blir styrkan på fluorescenssignalen olika stark. Dessa signaler talar sedan om vilka gener som är på eller av i en viss cell. Höga signaler tyder på högt uttryck av en gen och låga signaler på lågt uttryck. Tekniken används inom olika områden, t.ex. inom cancerforskning där olika cancertyper screenas för att hitta gener som är påverkade.

I min avhandling har jag använt mig av microarrayer för att studera genuttrycket i B- och T-lymfocyter. Lymfocyter är en typ av vita blodkroppar som behövs för vårt immunförsvar. B-lymfocyterna mognar ut till plasmaceller och deras främsta roll är att producera antikroppar (immunoglobuliner) med bestämd specificitet. B-cellerna används även för att presentera främmande ämnen (antigen) för T-lymfocyter. T-lymfocyter delas upp i T- hjälparceller och cytotoxiska T-celler. T-hjälparceller bidrar till aktivering av andra lymfocyter vid ett immunsvar medan cytotoxiska T-celler har

förmåga att skada eller döda andra celler. Lymfocyterna karakteriseras av olika ytmarkörer; CD19 för B-celler, CD3 för T-celler, CD8 för cytotoxiska T-celler och CD4 för T-hjälparceller. T-hjälparcellerna (CD4) kan vidare delas in i T-hjälpar 1 (Th1) och 2 (Th2) celler beroende på vilken typ av ämne (cytokin) de producerar.

De lymfocyter jag har undersökt har saknat några viktiga molekyler som tagits bort genom s.k. "utknockning". B-cellerna har saknat ett proteinkinas (enzym) som heter Bruton's tyrosinkinas (Btk) och som är viktig för B-cellernas utveckling. Vid avsaknad av funktionellt Btk, ofta genom olika mutationer, får man som människa en primär immunbristsjukdom som heter X-länkad agammaglobulinemi (XLA), vilket innebär defekt B-cellsutveckling och i och med det avsaknad av livsviktiga antikroppar. Pga. att sjukdomen är X-länkad kan bara pojkar bli sjuka, flickor kan vara bärare av sjukdomen men de blir inte sjuka av den. Jag har använt mig av två musmodeller som liknar sjukdomen hos människa. I den ena musmodellen har Btk tagits bort (knockats ut) och i den andra finns en mutation (R28C) i en del av Btk-genen. Sjukdomen hos möss, där mutationen R28C sker, kallas X-länkad immunbristsjukdom (Xid). Vi har undersökt och jämfört genuttrycket i dessa två modeller mot friska möss genom att köra microarrayer (Artikel I och II).

Btk tillhör en större familj av proteinkinaser där bl.a. Tec och Itk ingår. Dessa två finns i T-lymfocyter. Itk spelar en roll vid T-cellers aktivering och funktion och är en viktig faktor när det gäller utvecklingen av vanliga T-celler (conventional). Om Itk saknas utvecklas nativa T-celler (innate), som man tror har en roll i det primära immunförsvaret. I artikel III har vi undersökt genuttrycket i ostimulerade samt stimulerade mus T-celler som saknar Itk. I artikel IV har vi gjort ännu fler jämförelser och nu med möss som saknar Itk, Tec och både Itk och Tec med och utan stimulering.

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