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Function of Tec Family Kinases in the Innate Immune
Response to Fungal Pathogens

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Summary

The human fungal pathogen *Candida albicans* (Ca) represents the 4th-most frequent cause of nosocomial bloodstream infections causing mortality rates of about 40%. Different risk factors contribute and facilitate infections with Ca: basal diseases (like HIV), as well as impaired immunity (prolonged stationary clinical therapy, patients in intensive care units, organ or bone marrow transplantations e.g.).

To mount a protective immune response against Ca, the innate immune system must rapidly sense and respond to invading pathogens. Through the stimulation of specific pattern recognition receptors (PRRs), immune cells trigger signal transduction pathways that orchestrate immune response activation, cytokine production and other effector functions, ultimately aiming to eliminate pathogens.

One group of intracellular signaling molecules involved in this process is the Tec kinase family of non-receptor tyrosine kinases (Bmx, Btk, Itk, Rlk and Tec), which are preferentially expressed in cells of the haematopoietic system, including innate immune cells (monocytes/macrophages, dendritic cells e.g.). Deficiencies in Tec family kinases cause several immunological defects in humans and mice, including defects in B cell and T cell function. However, very little is known about the role and function of Tec family kinases in innate immune cells and nothing is known about their possible role in fungal-host interactions.

Here, we show that mouse innate immune cells of Tec kinase-deficient mice show cell type-specific changes in the immune response to *Candida*. Primary bone marrow-derived macrophages from Tec kinase knock-out mice exhibit reduced Ca killing capacity, diminished production of inflammatory cytokines and reactive oxygen species (ROS) upon infection with *Candida*. In contrast, bone marrow-derived myeloid dendritic cells show changes in the cytokine response, possibly altering T cell polarization which in turn influences the outcome for the host.

Moreover, *in vivo* experiments in a mouse model of disseminated candidiasis revealed improved survival of Tec^{-/-} mice. Fungal burdens in kidneys are altered and implicate a preferential induction of a tolerogenic immune response to *Candida albicans*.

For the first time, we are able to show that Tec Kinases are involved in the innate immune response of fungal infections in a cell type-specific manner. Additionally, we provide evidence that Tec Kinases play a role in a Ca i.v. challenge model in mice.

Zusammenfassung

Der human-pathogene Pilz *Candida albicans* (Ca) ist momentan der vierthäufigste Krankenhauskeim. Systemische Infektionen mit *Candida albicans* zeigen eine Mortalitätsrate von etwa 40%. Unterschiedliche Risikofaktoren tragen zur Infektion bei: sowohl basale Krankheiten (etwa HIV) als auch ein geschwächtes Immunsystem (lange Therapie, Patienten in Intensivstationen, Organ und Knochenmarks-Transplantationen, etc.).

Das effektive Erkennen und Einschreiten der angeborenen Immunität ist essentiell, um eine schützende Immunantwort gegen Ca zu generieren. Die Stimulation von Mustererkennungs-Rezeptoren (PRRs) führt zur Anschaltung von Signalwegen und diese zur Aktivierung des Immunsystems, der Zytokin-Produktion und vieler anderer Zell-Effektor-Funktionen. Dies alles trägt zur Eliminierung eines Pathogens bei.

Eine wichtige Gruppe intrazellulärer Signal-Kinasen stellen die nicht rezeptor-assoziierten Tyrosinkinasen der Tec Familie (Bmx, Btk, Itk, Rlk und Tec) dar. Mitglieder dieser Familie werden hauptsächlich in Zellen des Hämatopoetischen-Systems exprimiert, somit auch in Zellen des angeborenen Immunsystems (Monozyten/Makrophagen, Dendritische Zellen, etc.). Der Verlust von oder Mutationen in Kinasen der Tec Familie verursachen unterschiedlichste Immundefekte bei Mensch und Maus, unter anderem Störungen in B-Zell und T-Zell Funktion. Interessanterweise ist über die genaue Rolle dieser Kinasen in Zellen des angeborenen Immunsystems sehr wenig bekannt und über eine etwaige Rolle der Kinasen während Pilzinfektionen ist bis zum heutigen Tag nichts berichtet worden.

Diese Arbeit berichtet zum ersten Mal über eine zelltyp-spezifische Rolle der Tec Kinasen in Infektionen mit Ca. Tec Kinasen-defiziente primäre Makrophagen aus dem Knochenmark zeigen bei Infektionen mit *Candida spp.* eine reduzierte Produktion von reaktiven Sauerstoff Molekülen (ROS), reduzierte Produktion von inflammatorischen Zytokinen und eine erhöhte Überlebensrate von Ca. Im Gegensatz dazu zeigen Tec Kinasen-defiziente primäre Dendritische Zellen aus dem Knochenmark ein verändertes Zytokin-Profil, welches große Bedeutung bei der Polarisierung von naiven T Zellen haben könnte und damit möglicherweise den Infektionsverlauf beeinflusst.

Erste *in vivo* Versuche mit Tec Kinasen-defizienten Mäusen zeigten außerdem eine deutlich verbesserte Überlebensrate von Tec $-/-$ Mäusen, veränderte Keimzahlen an Ca in Nieren und eine mögliche Induktion einer starken tolerogenen Immunantwort.

Diese Arbeit bestätigt daher zum ersten Mal die *in vitro* und *in vivo* Rolle von Kinasen der Tec Familie bei Pilzinfektionen mit *Candida albicans*.

Table of contents

1	Introduction	1
1.1	The Immune System of Mammals	1
1.1.1	<i>The innate immune system.....</i>	<i>1</i>
1.1.2	<i>The adaptive immune system.....</i>	<i>2</i>
1.1.3	<i>T cell – mediated immunity.....</i>	<i>3</i>
1.2	The Tec Kinase Family.....	6
1.2.1	<i>Structure and function of Tec family kinases.....</i>	<i>6</i>
1.2.2	<i>Tec family kinases in health and disease.....</i>	<i>9</i>
1.3	Candida spp. – Opportunistic Fungal Pathogens	10
1.3.1	<i>Candida albicans.....</i>	<i>11</i>
1.3.2	<i>Candida glabrata.....</i>	<i>11</i>
1.4	Immunity to Candida spp.	12
1.4.1	<i>PRRs and innate antifungal responses.....</i>	<i>14</i>
1.4.2	<i>A mouse model to study systemic candidiasis</i>	<i>15</i>
	Aims of the Thesis	16
2	Materials and Methods	17
2.1	Fungal Methods	17
2.1.1	<i>Strains.....</i>	<i>17</i>
2.1.2	<i>Culture media for Candida species.....</i>	<i>17</i>
2.1.3	<i>Growth of Candida species.....</i>	<i>18</i>
2.1.4	<i>Heat-killing of Candida albicans.....</i>	<i>18</i>
2.1.5	<i>UV-killing of Candida species.....</i>	<i>18</i>
2.1.6	<i>Alexa Fluor 488-labeling of Candida species.....</i>	<i>19</i>
2.2	Mammalian Cell Culture	19
2.2.1	<i>Primary macrophages.....</i>	<i>19</i>
2.2.1.I	<i>Primary culture of bone marrow-derived macrophages (BMDMs).....</i>	<i>19</i>
2.2.1.II	<i>Preparation of L-conditioned media.....</i>	<i>20</i>
2.2.1	<i>Primary dendritic cells.....</i>	<i>21</i>
2.2.1.I	<i>Primary culture of myeloid dendritic cells (mDCs).....</i>	<i>21</i>
2.2.1.II	<i>Preparation of X-conditioned media.....</i>	<i>22</i>
2.2.3	<i>Primary neutrophils (PMNs).....</i>	<i>22</i>

2.3	Candida-Host Interactions	24
2.3.1	<i>Experimental setup for interaction experiments</i>	24
2.3.1.1	<i>Candida-BMDM interactions</i>	24
2.3.1.2	<i>Candida-mDC interactions</i>	24
2.3.1.3	<i>Candida-PMN interactions</i>	24
2.3.2	<i>Endpoint dilution killing assay</i>	25
2.3.3	<i>Phagocytosis assay</i>	25
2.3.4	<i>Apoptosis assay</i>	26
2.3.5	<i>Reactive oxygen species (ROS) assay</i>	26
2.3.6	<i>FACS – staining of immune cells</i>	27
2.4	Protein Methods	27
2.4.1	<i>Protein samples from mammalian cells</i>	27
2.4.2	<i>SDS-polyacrylamid gel electrophoresis (SDS-PAGE)</i>	28
2.4.3	<i>Western Blotting</i>	29
2.4.3.I	<i>Semi-dry transfer of proteins</i>	29
2.4.3.II	<i>Immunodetection</i>	29
2.4.3.III	<i>Stripping of immunoblots</i>	30
2.4.4	<i>Enzyme-linked immunosorbent assay (ELISA)</i>	30
2.4.4.I	<i>Plate-ELISA</i>	30
2.4.4.II	<i>Bead-ELISA</i>	31
2.5	RNA Methods	32
2.5.1	<i>RNA extraction from mammalian cells & mouse tissue</i>	32
2.5.2	<i>Reverse transcription of RNA samples</i>	32
2.5.3	<i>Real-time polymerase chain reaction (RT-PCR)</i>	33
2.6	<i>In Vivo</i> Candida Infection Studies	34
2.6.1	<i>Mice</i>	34
2.6.2	<i>Systemic Candida infection - Survival</i>	34
2.6.3	<i>Systemic Candida infection</i>	34
2.6.3.I	<i>Determining fungal burden</i>	34
2.6.3.II	<i>Restimulation of primed splenocytes</i>	35
2.6.3.III	<i>Characterization of splenocytes</i>	35
2.6.3.IV	<i>Isolation and restimulation of T cells</i>	37
2.6.3.V	<i>Dendritic cell (mDC) / T cell co-culture</i>	38
2.6.3.VI	<i>Urea in blood (BUN)</i>	38
2.7	Statistics	38

3	Results	39
3.1	In Vivo Role of Tec Family Kinases in anti-fungal Responses.....	39
3.1.1	<i>Survival of mice infected with Candida albicans</i>	39
3.1.2	<i>Mice challenged with Candida albicans</i>	41
3.1.3	<i>Restimulation of splenocyte cultures</i>	43
3.2	Functional Characterization of Tec Family Kinase-deficient Immune Cells...53	
3.2.1	<i>Analysis of bone marrow of Tec Kinase-deficient mice</i>	53
3.2.2	<i>Tec family kinases and their role in BMDMs</i>	55
3.2.2.1	<i>Phagocytosis of Candida spp.</i>	55
3.2.2.2	<i>Production of reactive oxygen species (ROS)</i>	56
3.2.2.3	<i>In vitro killing of Candida albicans</i>	57
3.2.2.4	<i>Induction of apoptosis</i>	58
3.2.2.5	<i>Cytokine and chemokine production in response to Candida spp.</i>	59
3.2.3	<i>Tec family kinases and their role in mDCs</i>	62
3.2.3.1	<i>Phagocytosis of Candida spp.</i>	62
3.2.3.2	<i>Activation of mDCs by Candida albicans</i>	63
3.2.3.3	<i>Cytokine production in response to Candida spp.</i>	64
3.2.3.4	<i>MAP kinase signalling in response to Candida spp.</i>	65
3.2.3.5	<i>STAT signalling in response to Candida spp.</i>	67
3.2.4	<i>Tec family kinases and their role in PMNs</i>	68
3.2.4.1	<i>In vitro killing of Candida albicans</i>	68
3.2.4.2	<i>Cytokine production in response to fungal stimuli</i>	69
3.2.5	<i>Tec family kinases and their role in T cells</i>	70
3.2.5.1	<i>Restimulation of primed T cells</i>	70
3.2.5.2	<i>Dendritic cell / T cell co-culture</i>	72
4	Discussion	75
5	References.....	84
6	Appendix.....	91

1 INTRODUCTION

1.1 The Immune System of Mammals

The mammalian immune system comprises one of the most complex structures found in living organisms. The intricacy of interacting cells, communicating signalling – pathways and essential fine-tuning during an immune response, are just at the beginning of being understood. Hence, the immune system takes over important physiological functions like the elimination of pathogenic microbes, but also the surveillance over abnormal and/or cancer cells and finally the removal of apoptotic and dead cells (Abbas et al., 2007).

Innate immunity represents the phylogenetically oldest mechanism to fight invaded pathogens and can be found in almost all multicellular organisms (therefore also in insects and plants). Following innate immunity, the “first line of defence”, the vertebrate immune system is able to mount another, in some cases even more powerful, fighting mechanism: the adaptive immunity (Abbas et al., 2007).

1.1.1 *The innate immune system*

The defence machinery of the innate immune system consists of cellular, as well as of biochemical mechanisms, which are operating at any time, and thus are able to respond to infections quickly. Components of the innate immune system include physical and chemical barriers (epithelia, antimicrobial substances), phagocytic cells (macrophages, neutrophils and granulocytes), natural killer cells (NK cells), the complement system and cytokines – signalling proteins that regulate immune effector functions. Cells of the innate immune system express germline-encoded pattern recognition receptors (PRRs), which are able to detect highly conserved and characteristic pathogen-associated molecular patterns (PAMPs). Notably, triggering an innate immune response can sometimes completely eliminate or kill an invaded pathogen. Nonetheless, cells of the innate immune system are also important activators and regulators of the adaptive immune system in most vertebrates (Abbas et al., 2007).

1.1.2 *The adaptive immune system*

As mentioned earlier, innate immunity has the advantage of being rapidly activated after infection. Nevertheless, not all pathogenic microbes are cleared successfully by an innate immune response. Therefore, additional defence mechanisms evolved: the adaptive immune system. The major advantages of adaptive immunity can be defined by two characteristics: adaptive immunity shows exceedingly high specificity for molecules present on pathogens, and the immune system is able to “recall” successful immune responses to fight an infection more vigorously upon repeated encounters with pathogens. Components of the adaptive immune system include lymphocytes and antigen presenting cells (APCs) and their cytokine products.

Adaptive immunity is able to fight infections in two different ways. Notably, the decision upon which of the two types is chosen, is highly depending on the nature of the encountered pathogen and the course of infection:

- Humoral immune responses are essentially used for clearance of extracellular pathogens and their toxins. Humoral immunity is mediated mainly by B lymphocytes and their secreted serum antibodies or mucosal secretions. Hence, these molecules are able to recognize microbial antigens (a pathogen itself but also its secreted toxins) and neutralize them or tag them for elimination by phagocytes.
- Cell-mediated immune responses are used for the elimination of intracellular microbes. Cellular immunity is mediated mainly by T lymphocytes, which are able to promote the killing of already infected cells or boost the killing-capacity of phagocytes present at the site of infection.

In general, while innate immune responses are activated within minutes after infection, adaptive immunity needs much more time (up to several days) to develop its whole defence capacity and armory.

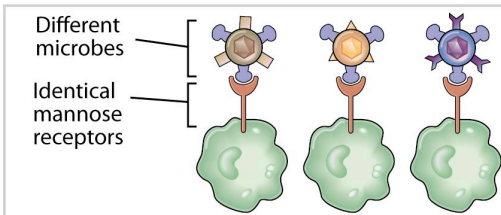
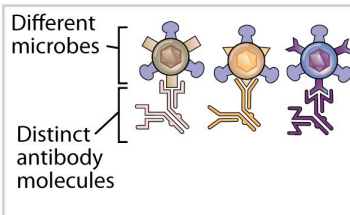
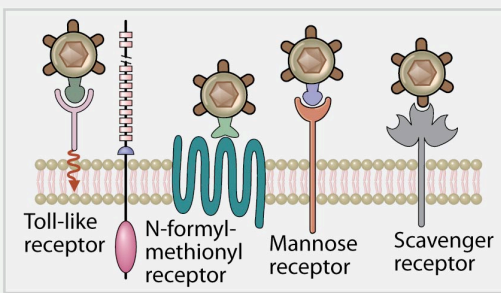
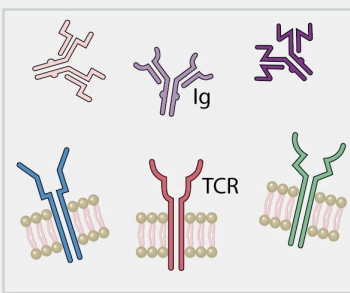
	Innate immunity	Adaptive immunity
Specificity	For structures shared by classes of microbes ("pathogen-associated molecular patterns")  Different microbes Identical mannose receptors	For structural detail of microbial molecules (antigens); may recognize nonmicrobial antigens  Different microbes Distinct antibody molecules
Receptors	Encoded in germline; limited diversity ("pattern recognition receptors")  Toll-like receptor N-formyl-methionyl receptor Mannose receptor Scavenger receptor	Encoded by genes produced by somatic recombination of gene segments; greater diversity  Ig TCR
Distribution of receptors	Nonclonal: identical receptors on all cells of the same lineage	Clonal: clones of lymphocytes with distinct specificities express different receptors
Discrimination between self and nonself	Yes; host cells are not recognized or they may express molecules that prevent innate immune reactions	Yes; based on selection against self-reactive lymphocytes; may be imperfect (giving rise to autoimmunity)

Figure 1.1: **Innate and adaptive immunity**

The major characteristics of the innate as well as the adaptive immune system are shown. (Abbas et al., 2007)

1.1.3 *T cell – mediated immunity*

To initiate and develop an effective adaptive immune response, respective PAMP-carrying pathogens must be phagocytosed quickly, and displayed to naïve T cells to stimulate a cell-mediated immune response. For this purpose, the immune system developed antigen-presenting cells (APCs), of which dendritic cells (DCs) represent the most specialized ones.

After phagocytosis of microbes, antigenic regions of destroyed pathogens are presented on the surface of dendritic cells via major histocompatibility complexes (MHC). Recognition of these antigens by specific T cell receptors (TCRs), as well as the cytokine environment provided by DCs, triggers activation and high proliferation of a specific T cell clone (clonal expansion). These events trigger production of

certain cytokines and the activation of cellular effector functions, ultimately leading to the elimination of the pathogen.

Both, CD4⁺ or CD8⁺ T cells can undertake effector functions of the cell-mediated immunity:

- CD8⁺ T cells are referred to as cytotoxic T cells (or cytolytic lymphocyte – CTL). These cells are able to actively lyse infected cells, including non-phagocytic cells, by releasing cytotoxins like granzymes, granulysin and perforin after their activation.
- CD4⁺ T cells are referred as helper T cells (Th) and can be divided into currently 4 major subfamilies (Th1, Th2, Th17 and Treg), each with specific functions. The decision of a naïve T cell to develop into a specific Th cell is highly dependent on the pathogen, and the cytokine environment provided by APCs at the site of infection (Figure 1.2). After differentiation into a lineage specific T cell, cells start to produce distinctive helper T cell cytokines, which then activate certain immune effector functions (e.g. cell recruitment, cell activation, inflammation). Thus, each Th-lineage is able to fight particular microbes very efficiently. Interestingly, many pathogens have acquired mechanisms blocking the development of a certain Th-lineage, enabling them to withstand cell-mediated immunity or bypassing immune surveillance (Abbas et al., 2007).

After successful elimination of a pathogen, a rapid decline of proliferated T cells occurs. This reduction is known as homeostasis and describes the mechanisms of regulated pathways leading to lymphocyte death (apoptosis) and inactivation. Some of the primed T cells become dormant, but they remain within the organism and are important in building up T cell memory, which allows the immune system to respond quickly and vigorously at repeated encounters with the same pathogen.

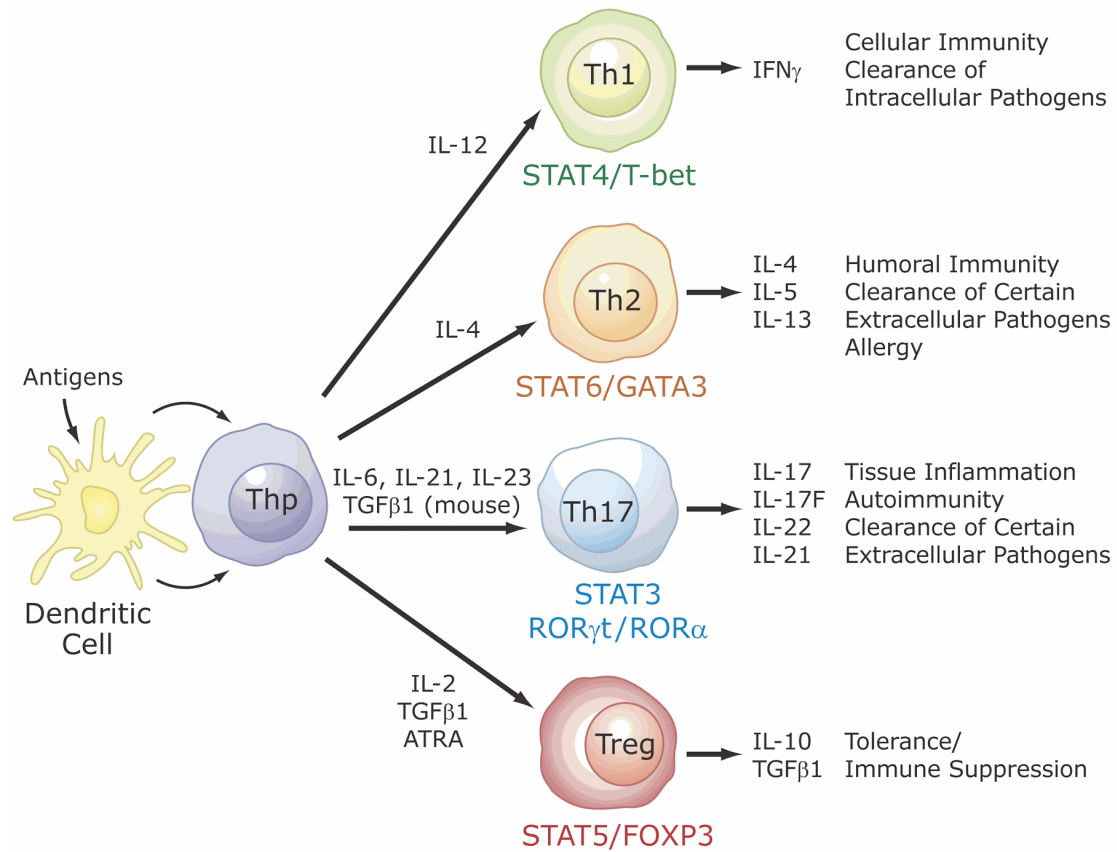


Figure 1.2: T helper cell-mediated immunity

Activation and priming of T cells requires APCs. APCs stimulate pluripotent T helper cell (Thp) and provide a cytokine environment, promoting T cell development into dedicated lineages. IL-12 induces the development of Th1 cells, the major producers of IFN γ . IL-4 environment leads to Th2 cells, which then secrete IL-4, IL-5 and IL-13. IL-6, IL-23 and TGF β -1 for Th17 cells, which mainly release IL-17, IL-17F, IL-22 and IL-21. IL-2 and IL-10 (not shown) are essential for the induction of a Treg response, which is characterized by the presence of large amounts of IL-10. For each Th-lineage, the essential STAT signalling protein as well as the unique transcription factor is depicted (Jetten, AM., 2009).

1.2 The Tec Kinase Family

The Tec kinase family represents the second-largest group of non-receptor tyrosine kinases and consists of 5 different members, which are found exclusively in vertebrates. Tec (tyrosine kinase expressed in hepatocellular carcinoma), Bmx (bone marrow kinase on the X chromosome, also known as Etk), Btk (Bruton's tyrosine kinase), Itk (IL-2-inducible T cell kinase, also known as Emt) and Rlk (resting lymphocyte kinase, also known as, Txk) are preferentially expressed in cells of the hematopoietic system. Interestingly, each cell type of the hematopoietic system expresses its own subset of Tec kinases. While some cells express just one specific member of the family, other cell types reveal expression of two to three different members. Whether different Tec kinases expressed in one cell share redundant functions or whether each kinase is responsible for particular cellular functions remains an open question (Köprülü D. and Ellmeier W., 2009).

1.2.1 *Structure and function of Tec family kinases*

Tec family kinases show a quite modular structure (see Figure 1.3) and full activation of the kinase is tightly regulated. Regulation mechanisms include subcellular localization, phosphorylation and subsequent autophosphorylation, colocalization with membrane-associated adaptor proteins and intra- as well as intermolecular interactions (Köprülü D. and Ellmeier W., 2009). Thus, activation of Tec family kinases represents a multi-step process, which has been studied to a great extent in T lymphocytes.

After activation of a membrane-receptor, Tec kinases are recruited to the membrane via the interaction of their pleckstrin homology (PH) domain with phosphatidylinositol (3,4,5)-triphosphate (PIP₃) produced by PI3-Kinase (PI3-K). Thereupon, Src family kinases phosphorylate a tyrosine residue located in the activation loop of the kinase domain, which leads to autophosphorylation within the Src homology domain 3 (SH3), leading to the full activation of the Tec kinases (Berg L. et al, 2005). Inter- and intramolecular interactions regulate activity of Tec family kinases. Intermolecular reciprocal interplay of TH and SH3 domains between two Tec kinase family members (especially shown for Tec and Btk) lead to the formation of homodimers (Berg L. et al, 2005). Intramolecular interactions of TH and SH3 domains, which prevent binding to ligands via those domains (Andreotti AH. et al, 1997), have been shown to negatively regulate activation of Tec family kinases.



Figure 1.3: **Structure of Tec famIL-y kinases**

The N-terminal pleckstrin homology (PH) domain represents the most characteristic feature of Tec famIL-y kinases with the exception of Rlk, which carries a palmitoylated cysteine-rich region. With this PH domain Tec kinases are able to get recruited to the plasma membrane by interacting with phospholipids and phosphorylated proteins. The PH domain is followed by a Tec homology (TH) domain, which comprises of a Btk homology (BH) domain and one or two proline-rich (PR) regions. The TH domain is followed by two Src homology domains (SH3 and SH2), which are essential for interaction with phosphorylated tyrosine residues or proline-rich regions, respectively.

Tec family kinases have been implicated in several distinct cellular functions (Figure 1.4): the intracellular mobilization of Ca^{2+} (Fluckiger AC. et al, 1998), the activation of integrins and changes in cell adhesion (Woods ML. et al, 2001 & Finkelstein LD. et al, 2005), the regulation of apoptosis (Uckun FM, 1998), the reorganization of the actin filament system (Grasis JA. et al, 2003), the migration processes of cells after chemokine stimulation (Takesono A. et al, 2004), the regulation of M-CSF signalling in monocytes and macrophages (Melcher M. et al, 2008), the regulation of phagocytosis (Jongstra-Bilen J. et al, 2008), the development of T cells (Berg LJ. et al, 2005), the involvement in TLR signalling (Jefferies CA. et al, 2003) and in effector functions of macrophages (Mukhopadhyay S. et al, 2002; Mangla A. et al, 2004).

Interestingly, many of the described cellular functions were studied in great detail in T cells or B cells. In contrast, the function of Tec family kinases in myeloid cells is just at the beginning of being understood.

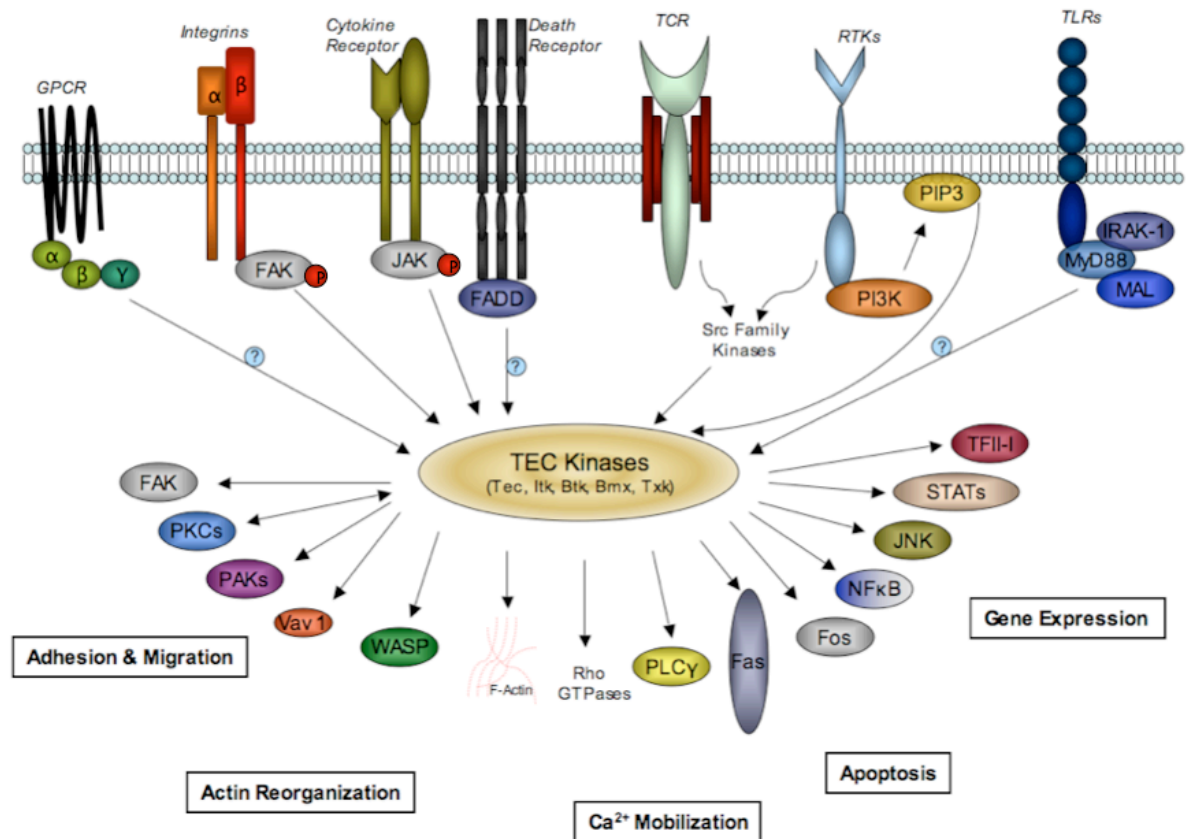


Figure 1.4: **Cellular functions associated with Tec family kinases**

Signalling partners of Tec family kinases and their respective cellular function are shown. Question marks indicate suggested but not directly proven interaction partners. GPCR: G-Protein coupled receptor; TCR: T cell receptor; RTK: Receptor tyrosine kinase; TLR: Toll-like receptor; FAK: focal adhesion kinase;

1.2.2 *Tec family kinases in health and disease*

Bruton's tyrosine kinase, Btk, is probably the most prominent Tec kinase family member. Patients showing mutations in the *BTK* gene locus (truncations, missense mutations) but also in *cis*-regulatory regions (Valiaho J. et al, 2006), suffer from "X-linked agammaglobulinemia" (XLA). XLA patients exhibit reduced levels of immunoglobulins (Igs) in sera due to defects in B lymphocyte development (Tsukada S. et al, 1993). Thus, no mature antibody-producing cells can be found within their organism. XLA-patients suffer from regular, and in many cases, life-threatening bacterial (encapsulated) infections. Such infections mainly affect surfaces of mucosal membranes, such as the middle ear, sinuses and lungs, but in some instances can also involve the bloodstream or internal organs (Winkelstein JA. et al, 2006).

In mutant mice, the single point mutation R28C in the *Btk* gene located within the PH domain of Btk, mirrors a much milder form of XLA, called X-linked immunodeficiency (*xid*) (Rawlings DJ. et al, 1993). Disruption of the whole *Btk* locus phenotypically resembles *xid* mice (Khan WN. et al, 1995). *Xid* mice display reduced levels of IgG3 and IgM in serum, lack peritoneal CD5⁺ B cells and exhibit reduced numbers of peripheral and IgM^{lo} IgD^{hi} B cells (Maas A. et al, 2001). Notably, isolated cells (e.g. monocytes) from either XLA patients or *xid* mice show phenotypical differences when stimulated (e.g. Horwood NJ. et al, 2006), suggesting that results in *xid* mice might not be necessarily be valid for XLA patients – even when interpreting cell-based *in vitro* assays.

Importantly, the role of Tec family kinases during infectious processes is currently poorly understood. Hence, there are only few reports dealing with this issue. *Xid* mice exhibit reduced *in vivo* clearance of microfilarial infections (Mukhopadhyay S. et al, 2002). Other studies suggested that *xid* mice are less susceptible to inflammatory collagen-induced arthritis (Jansson L. et al, 1993) and autoimmune diseases such as autoimmune encephalomyelitis (Svensson L. et al, 2002). Itk has been reported to be essential in mounting a proper Th2 response in parasite infection models (*Nippostrongylus brasiliensis* or *Schistosoma mansoni*; Fowel DJ. et al, 1999) and to be crucial for CD8⁺ T cell function in viral infections, including lymphocytic choriomeningitis virus (LCMV), vaccinia virus (VV) and vesicular stomatitis virus (VSV) (Bachmann MF. et al, 1997). Recently, it has been shown that Tec ^{-/-} mice display a CD44^{high} CD62L⁻ Th17 subset, which is responsible for higher production of interleukin 17 and enhanced clearance of *Streptococcus pneumoniae* in the lung of these mice (Boucheron N. et al, 2010).

1.3 *Candida spp.* – Opportunistic Fungal Pathogens

In humans, infections with fungi can cause superficial, cutaneous, subcutaneous as well as systemic and allergic diseases. Two main types of fungal infections are known: First, true pathogenic fungal infections – in this case the fungus is virulent regardless of the host's medical condition (*Coccidioides*, *Paracoccidioides*, *Histoplasma*). Second, opportunistic fungal mycoses – manifestation of the disease is dependent on host's medical condition. Fungi, causing opportunistic infections, are normally of low virulence (e.g. *Aspergillus*, *Cryptococcus*, *Candida spp.*) (Kullberg BJ. et al, 2002).

Candida species are present at any time as part of the residential microbial flora. Therefore, *Candida spp.* represent a typical commensal colonizer, which can be found in the gastrointestinal as well as in the genitourinary tract, on most mucosal layers and to a lesser extent on the skin. However, in basal diseases (like HIV) or under impaired immunity (prolonged stationary clinical therapy, intensive care unit patients, organ or bone marrow transplantation, chemotherapy, elderly as well as newborn individuals), *Candida* species are able to become opportunistic pathogens. *Candida spp.* constitute the 4th-most frequent cause of nosocomial bloodstream infections with a mortality rate of about 40% (Pfaller MA. and Diekema DJ., 2007). Incidences of infections with *Candida spp.* keep rising due to the increased abundance of immunosuppressive therapies and the excessive use of broad-spectrum antibiotics. Briefly, *Candida* infections can be categorized into two different classes:

- Superficial infections: This kind of infection mainly affects mucosal tissues and, in general, represents a non life-threatening disease. Thus, non-immunocompromised patients are able to efficiently counter *Candida spp.* during superficial infections.
- Invasive candidiasis: In invasive or systemic candidiasis *Candida spp.* were able to enter the blood stream (e.g. due to implanted surgical devices or, especially, through central venous catheters). This allows *Candida* to spread to one or multiple organs within the patient ("pathological niche"), representing a life-threatening contagion. The major target-organs for *Candida spp.* disseminations in humans are kidneys, lung, brain as well as the digestive tract (Knox WF. et al, 1987 and Tuite A. et al, 2004)

1.3.1 *Candida albicans* (Ca)

Candida albicans is an obligatory diploid fungus and represents the most frequent causative agent of candidiasis in human (Verduyn L. et al, 1999). Depending on environmental conditions, Ca shows three different morphological shapes: the classical yeast, pseudohyphae and true hyphae (Figure 1.5). Hence, the ability to adapt various morphological shapes, promotes virulence of Ca, since mutant strains, unable to undergo morphological changes, are generally less virulent (Berman J. and Sudbery PE., 2002). The usage of the yeast-shape allows Ca to quickly disseminate within an organism by utilizing the blood stream as a “shuttle”. In contrast, the hyphal shape allows Ca to withstand phagocytosis mainly mediated by macrophages and neutrophils, whereas switching of the morphology after phagocytosis allows Ca the rupture of immune cells. Additionally, the expression of certain cell wall proteins in hyphal Ca-cells facilitates adhesion to host tissues and is therefore very important for tissue invasion. Other virulence factors implicated in Ca infections are the expression of adhesion molecules (early stages of infection), as well as the production and secretion of hydrolytic proteinases and lipases, which damage the host tissue (late stages of infection).

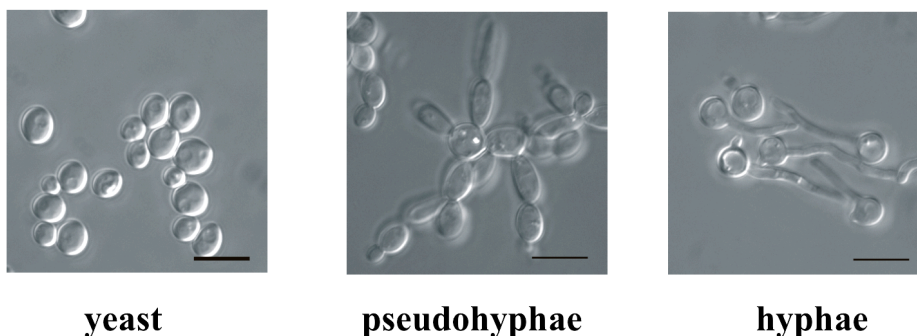


Figure 1.5: **Morphologic shapes of *Candida* spp.**

Yeast, pseudohyphal and hyphal shapes of *Candida albicans* are shown.

1.3.2 *Candida glabrata* (Cg)

Candida glabrata is a haploid fungus and represents the second to third-most common causative agent of candidiasis in human (Fidel PL. et al, 1999). Notably, Cg is unable to undergo morphological switches. Therefore, the yeast form embodies a commensal as well as a pathogen. Hence, *Candida glabrata* must have evolved different/other virulence mechanisms than *Candida albicans* to successfully persist in host tissues. Importantly, infections with Cg are often difficult to treat, since many strains show intrinsic resistance to different azole antifungal drugs (Pfeller MA. and Diekema DJ., 2007).

1.4 Immunity to *Candida* spp.

The manifestation and severity, the dissemination or clearance of fungi during the course of fungal infections is influenced mainly by the host's immune response. Therefore, various host defence mechanisms are triggered, ranging from innate to adaptive immunity.

Innate immunity constitutes one of the major components of the response to *Candida* infections, including simple barrier function of epithelial and mucosal surfaces in respiratory, gastrointestinal and genitor-urinary tract (Romani L., 2004) and cellular effector functions mediated by phagocytes like macrophages, neutrophils and dendritic cells (Mansour MK. and Levitz SM., 2002). Cellular effector functions include phagocytosis of *Candida*, followed by destruction of fungal cells via the respiratory burst and oxidative stress. Additionally, the secretion of microbicidal substances and the release of pro-inflammatory cytokines and chemokines, together with presentation of fungal antigens by APCs, are essential elements in the induction of an adaptive immune response (Romani L., 2004; MacCallum DM. et al, 2009; MacCallum DM. et al, 2009). Notably, a balance of pro- and anti-inflammatory cytokines produced by immune cells is crucial for the outcome of a *Candida* infection (Figure 1.6). Knock-out mice lacking either IFN- γ , TNF- α and IL-12 (pro-inflammatory) or IL-4 and IL-10 (anti-inflammatory) show changed susceptibilities to candidiasis *in vivo*, suggesting an essential role of inflammatory mechanisms during the host response to *Candida albicans* (Netea, MG. et al, 1999; Kaposzta, R. et al, 1998 and Tonnetti L. et al, 1995; Jouault T. et al, 2009).

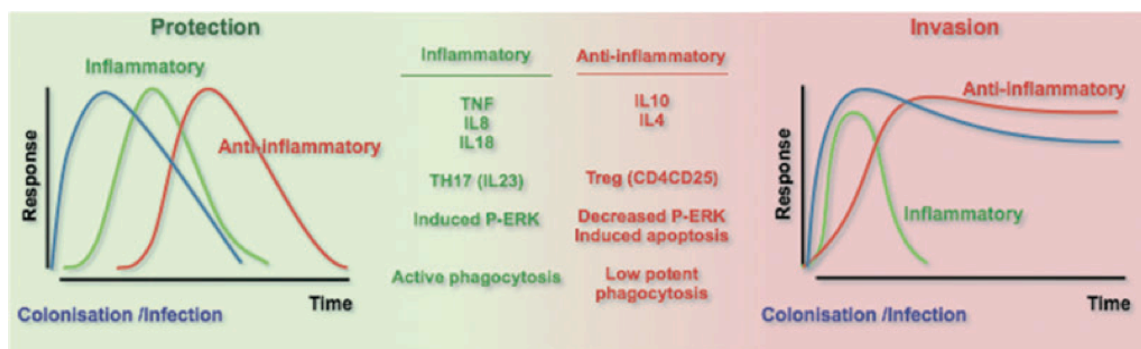


Figure 1.6: Initial immune response modulates protection or invasion of *Candida albicans*

Candida albicans activates inflammatory as well as anti-inflammatory mechanisms, leading to either protection or invasion against/of Ca. The importance of the kinetics and strengths of these responses are shown. An unbalanced anti-inflammatory response favours the development of disseminated candidiasis (Jouault T. et al, 2009).

The role of T cell-mediated immunity during *Candida* infections is currently poorly understood. An inflammatory Th1 response seems to be protective, while the induction of an immunosuppressive Th2 response seems to be detrimental for the host (Romani L., 2004). Recently, Th17 cells came into the focus of the *Candida* community, suggesting a pivotal role of Th17 responses during disseminated *Candida* infections (Lin Lin et al, 2009). Due to a high Th1/Th17-driven tissue inflammation, regulatory T cells (Treg) are believed to control and limit tissue damage by the release of large amounts of IL--10 (Netea MG. et al, 2004). Hence, the current picture of T cell-mediated immunity suggests an Th1/Th17-driven inflammatory response, which allows for clearance of the fungus, with pivotal contributions of Treg, as anti-inflammatory “guards”.

The role of humoral immunity and, therefore, the role of antibodies in fungal infections constitute a quite paradoxical situation. Antibodies promote binding (opsonization) and recognition of *Candida spp* by complement, thus, facilitating phagocytosis of fungal cells. Additionally, they prevent adherence of *Candida*, neutralize toxins and activate the complement system. However, the protective role of antibodies during *Candida* infections has been a matter of controversy due to the presence of specific antibodies in patients with systemic candidiasis (Casadevall A. et al, 2002; Han Y. and Cutler JE., 1995 and Matthews R. and Burnie J., 2001).

1.4.1 PRRs and innate antifungal responses

Toll-like receptors

The role of Toll-like receptors (TLRs) in antifungal immune responses has been described first by Lemaitre et al., showing that *Drosophila* flies deficient of *toll* were immediately overgrown by *Aspergillus fumigatus* (Lemaitre B. et al, 1996). In mammalian cells, TLRs have been associated with the recognition of fungal PAMPs. They trigger subsequent activation of intracellular kinase cascades, like the MAP-kinase pathway, as well as the activation of different transcription factors, like NF- κ B (Akira S. et al, 2006). Currently, at least 5 different TLRs seem involved in immune responses against *Candida spp.*, including TLR2 and TLR4 (Netea MG. et al, 2002), TLR6 (Netea MG. et al, 2008), TLR9 (van de Veerdonk FL. et al, 2008) and TLR7 (Bourgeois C. et al, 2011). While TLR2 seems to be linked to anti-inflammatory responses and the induction of Tregs, TLR4, TLR6 and TLR9 are implicated in pro-inflammatory signaling events. TLR7 was reported to be important for mounting a Type I interferon response.

C-type lectin receptors

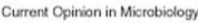
The C-type lectin receptors (CLRs) dectin-1, dectin-2, macrophage mannose receptor (MR), galectin-3, dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) and mincle recognize fungal PAMPs. The activity of selected members seems to be calcium dependent (e.g. dectin-2, mincle, DC-SIGN and MR).

Dectin-1 is predominantly expressed in myeloid cells and is stimulated by beta-1,3-linked glucans (Reid DM. et al, 2009). Dectin-1 signalling involves Syk and Src kinases, as well as CARD9 and both the canonical- and non-canonical NF- κ B pathways, as well as the NFAT transcription factor (Gringhuis SI. et al, 2009), thus, leading to the production of inflammatory cytokines and reactive oxygen species (ROS) (Skrzypek F. et al, 2009).

Dectin-2 mainly recognizes high mannose structures present on fungi and has been associated with the induction of Th17 responses during *Candida* infections. (Robinson MJ. et al, 2009).

Mincle is involved in recognition of *Candida albicans* by macrophages. It recognizes N-linked mannans and is mainly located in phagocytic cups (Wells CA. et al, 2008). Like many other CLRs, mincle induces inflammatory cytokines and chemokines (Yamasaki S. et al, 2008).

Galectin-3 is stimulated by beta-1,2-linked mannosides and has been reported to collaborate with TLR2 (Jouault T. et al, 2006).



Major fungal PAMPs and their respective PRRs, including their intracellular signaling cascades, are shown. ROS: reactive oxygen species; cPLA₂α: cytosolic phospholipase A₂α; MR: macrophage mannose receptor. (Bourgeois C. et al, 2010)

Mouse models constitute the preferred model to study systemic *Candida* infections. Thus, different infection routes can be chosen to infect mice: intravenous (i.v.), intraperitoneal (i.p.), intranasal and per os (p.o.) (de Repentigny L., 2004). Noteworthy, *Candida spp.* are not commensal colonizers of mice.

Aims of this Diploma Thesis

The main aim of this thesis was to answer the question whether Tec kinase family members are implicated in the immune response to fungal pathogens (*Candida spp.*), to investigate cell-type specific changes in host-fungal interactions and, if possible, to unravel possible molecular mechanisms of Tec family kinases during fungal pathogenesis.

As mentioned in 1.2 Tec kinases are expressed in cells of the hematopoietic system. Macrophages/monocytes express the family members Tec, Btk and Bmx and myeloid dendritic cells express Btk and Tec. Interestingly, very little is known about the role of these kinases in these cell lines and nothing is known about their role in fungal infections. We therefore wanted to address three major questions:

- I) Do Tec family kinase-deficient bone marrow-derived macrophages / myeloid dendritic cells show changed effector functions in response to *Candida spp.*?
- II) Do Tec family kinase-deficient myeloid dendritic cells show changed cytokine environment and altered T cell polarization *in vitro* in response to *Candida spp.*?
- III) Do Tec kinase family-deficient mice show altered susceptibilities to *Candida albicans in vivo*?

2 MATERIALS AND METHODS

2.1 Fungal Methods

2.1.1 Strains

Name	<i>Candida sp.</i>	Background strain	Genotype	Source or Reference
SC 5314	<i>C. albicans</i>	NA	Clinical isolate	(Gillum et al., 1984)
ATCC2001	<i>C. glabrata</i>	NA	Clinical isolate	18633: J. Infect. Dis. 1: 98, 1917.

NA = not applicable

2.1.2 Culture media for *Candida* species

Liquid media

1x YPD 10g/l Yeast extract (Difco)
 20g/l Bacto-Peptone (BD)
 2% (w/v) Glucose

Yeast extract and peptone were dissolved in ddH₂O and autoclaved. Glucose was dissolved in ddH₂O (20% glucose stock-solution) and added to sterile YP-medium to get the final concentration of 2%.

YPD plates

2x YPD 20g/l Yeast extract (Difco)
 40g/l Bacto-Peptone (BD)
 4% (w/v) Glucose

Yeast extract and peptone were dissolved in 200ml ddH₂O and autoclaved. Finally, 50ml sterile 20% glucose stock-solution and 250ml 4% autoclaved agar (w/v), molten in a microwave, were added and plates were poured (sterile, 9 cm round dishes).

2.1.3 Growth of *Candida* species

- Desired *Candida* strain was streaked out on YPD – plates using –80°C fungal stocks.
- A single *Candida* colony was picked and grown to saturation overnight in 5ml YPD with continuous shaking at 30°C.
- On the next morning, the overnight culture was diluted to 0.2 OD₆₀₀ in 20ml YPD and incubated with continuous shaking at 30°C until the culture reached 1 OD₆₀₀.

Preparation of fungi for host interaction experiments

- 20ml of a *Candida* culture (OD₆₀₀ ~1) were centrifuged at 1200xg for 5min, at room temperature and the resulting pellet was resuspended in 30ml sterile room temperature PBS (repeated twice).
- The washed fungal pellet was resuspended in 4ml sterile PBS and the fungal cell counts/ml were determined (CASY-Cellcounter).
- For *in vitro* infection experiments, fungal suspensions were diluted in pre-warmed (37°C) high-glucose DMEM, so that fungus-mammalian cell co-culture is performed at a ratio of 2 fungal cells per 1 mammalian cell (multiplicity of infection (MOI) 2:1).
- For *in vivo* experiments, fungal suspensions were diluted in sterile PBS to desired densities, recounted with the CASY-Cellcounter. As controls, colony forming units (CFUs) were determined by plating the fungal suspensions on YPD-plates.

2.1.4 Heat-killing of *Candida albicans*

- A suspension of *Candida albicans* in DMEM (prepared as in section 2.1.3) was incubated at 65°C for 10min with continuous shaking. Viability was determined by plating the heat-killed suspension on YPD-plates.

2.1.5 UV-killing of *Candida* species

- A *Candida* suspension was prepared as described in section 2.1.3.
- About 1 x10⁷ cells/ ml fungi dilution in sterile PBS were transferred into a 10cm cell culture-treated Petri dish.
- The Petri dish was placed into in a UV crosslinker (Stratagene) and fungi were radiated at 950µJ/cm²
- Viability was determined by plating the UV-killed suspension on YPD-plates.

2.1.6 Alexa Fluor 488-labeling of *Candida* species

- 20ml of an 1 OD₆₀₀ *Candida* culture were centrifuged at 1200xg for 5min at room temperature; the resulting pellet was resuspended in 30ml sterile PBS (repeated twice) at RT.
- Cells were resuspended in 1ml 100mM HEPES buffer pH 7,5
- For staining, 100µl Alexa Fluor 488 (Invitrogen; 100mg/ml) were added to 900µl *Candida* culture and incubated for 60min at 30°C with continuous shaking in the dark.
- After staining, cells were centrifuged at 1200xg for 5min at room temperature, supernatant was discarded and cells were washed 3 times with 100mM HEPES buffer.
- Cells were finally resuspended in 100mM HEPES buffer and counted with CASY-Cell counter.

2.2 Mammalian Cell Culture

2.2.1 Primary macrophages

I) Primary culture of bone marrow-derived macrophages (BMDMs)

- Tibias and femurs from 8-10 week old mice (wild type or knock out mice) were dissected, quickly rinsed in 70% ethanol and placed in 15ml ice-cold sterile PBS. When all the limbs were collected, they were transferred in fresh 15ml ice-cold sterile PBS and kept on ice.
- To flush out the bone marrow, femurs were separated from tibias at the knee joint. Holding the bone with forceps above a sterile dish, cut off one extremity of the bone and using a 20ml syringe with a 27GX3/4 needle, bones were flushed with DMEM with 10% FCS, 100 U/ml Penicillin and 100µg/ml Streptomycin, into the medullary cavity until no more cells came out.
- Cell Suspension was then centrifuged at 300xg for 7min at 4°C.
- The resulting cell-pellet was resuspended in 2ml L-conditioned Media (BMDM media) and 1ml of this suspension was added to 7ml of media in a cell-culture treated plate (10cm square dish).
- Cells were incubated at 37°C and 5 % CO₂.
- On day 2, 5ml of BMDM media were added to the culture.

- On day 6-7, when the plate was getting confluent, media was aspirated (containing non-adherent cells), and cells were collected by gently scrapping plates with a soft rubber spatula. Cell suspension was centrifuged (300xg for 5min at room temperature), resuspended in BMDM-media and re-plated at a ratio of 1:2-1:4 in a square 10x10cm Petri dish. Cells were incubated at 37°C and 5 % CO₂ atmosphere.
- Media was changed completely every 2-3 days.
- After 10-12 days of culture, BMDMs were ready to be used for interaction experiments.

<u>BMDM media</u>	Dulbecco's modified Eagle's Medium DMEM, high glucose (4.5g/l), with L-Glutamine, without pyruvate (PAA) + 10% FCS + 100 U/ml Penicillin + 100µg/ml Streptomycin + 15-20% L-conditioned media
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II) Preparation of L-conditioned media

- Confluent 10cm dishes of CSF-1 producing L929 cells (ATCC# CCL-1) were splitted into 175cm²-flasks with 50ml/flask of high-glucose DMEM supplemented with 10% FCS without antibiotics.
- After 36-48 hours (cells were approximately 70% confluent), media was aspirated and replaced with 100ml/flask of starvation media (high-glucose DMEM without FCS and antibiotics).
- After 10 days, conditioned media was collected and filtered through Steritop 0.22µm GP express PLUS membranes (Millipore, Billerica, MA, USA). Media was stored frozen in 250-500ml aliquots at -20°C. A small aliquot was kept at 4°C for testing.

Testing L-conditioned media

- The bones of one mouse were flushed as described in section 2.2.1.I, and resulting cells were divided into five 10cm square Petri dishes.
- Cells were grown in 12ml of BMDM media supplemented with 0% - 20% of the fresh batch of L-conditioned media. As a positive control, same cells were grown in BMDM media supplemented with the optimal concentration of an old working batch of L-conditioned media.
- Media was changed completely every 2-3 days.
- After 6-7 days, cells were split 1:3 into fresh medium.

- After 10-12 days, the optimal concentration of new batch L-conditioned media was determined (assessing the rate of cell-proliferation).

2.2.2 *Primary dendritic cells*

I) Primary culture of myeloid dendritic cells (mDCs)

- Tibias and femurs from 8-10 week old mice (wild type or knock out mice) were dissected, quickly rinsed in 70% ethanol and placed in 15ml ice-cold sterile PBS. When all limbs were collected, they were transferred in fresh 15ml ice-cold sterile PBS and kept on ice.
- To flush out the bone marrow, femurs were separated from tibias at the knee joint. Holding the bone with forceps above a sterile dish, cut one extreme end of the bone; using a 20ml syringe with a 27GX3/4 needle, bones were flushed with DMEM with 10% FCS, 100 U/ml Penicillin and 100µg/ml Streptomycin, into the medullary cavity until no more cells came out.
- Cell suspension was then centrifuged at 300xg for 7min at 4°C.
- Resulting pellet was treated with 1ml Red Cell Lysis Buffer (incubation: 2 Min at RT).
- After lysis, suspension was centrifuged at 300xg for 7min (4°C). The white pellet was resuspended in 4ml mDC media. 1ml of this suspension was added to 7ml of mDC media (10cm round Petri dishes).
- Cells were incubated at 37°C and 5 %CO₂ atmosphere.
- On day 3 after isolation, additional 5ml of mDC-media were added.
- After 7 days of culture, mDC aggregates were collected by gently flushing media against the well wall to avoid detaching strongly adherent cells.
- Flushed mDC-cells were collected and were ready to be used for interaction experiments.

<u>mDCs media</u>	Dulbecco's modified Eagle's Medium DMEM, high glucose (4.5g/l), with L-Glutamine, without pyruvate (PAA)
	+ 10% FCS
	+ 100 U/ml Penicillin
	+ 100µg/ml Streptomycin
	+ 5-10% X-conditioned media

Red Blood Cell (RBC) lysis buffer 8.3g/l NH₄Cl in 0.01M Tris-HCl buffer (pH 7,0)

II) Preparation of X-conditioned media

- GM-CSF producing X-63 cell line (Zal et al., 1994) was grown in 10ml high-glucose DMEM supplemented with 10% FCS, 100U/ml Penicillin and 100µg/ml Streptomycin in a 75cm² flask until the cell suspension was dense.
- The cell suspension was diluted 1:2 with fresh high-glucose DMEM, supplemented with 10% FCS, 100 U/ml Penicillin and 100µg/ml Streptomycin.
- When cells became dense again, 30ml starvation media (high-glucose DMEM without FCS and antibiotics) were added, and cells were grown until reaching 100% confluency.
- Cells were transferred into a 250ml flask and 50ml fresh starvation media was added.
- When cells were dense again, additional 100ml starvation media was added.
- After 8-10 days of culturing, when most cells were dead, X-conditioned media was collected, centrifuged at 1000xg for 5min to pellet X-cells, and processed as described above in section 2.2.1.II for sterile-filtration of supernatants, storage and testing.

2.2.3 *Primary neutrophils (PMNs)*

- A discontinuous Percoll gradient was prepared as follows:
9ml Percoll were mixed with 1ml 10x PBS (= 100% isotonic Percoll solution).
440µl PBS were mixed with 1560µl 100% Percoll (= 78% solution).
620µl RPMI-full were mixed with 1380µl 100% Percoll (= 69% solution).
960µl PBS were mixed with 1040µl 100% Percoll (= 52% solution).
- Femurs and tibias of 3-4 mice (wild-type or knock-out mice) were taken and stored in ice cold full-RPMI. Bone marrow of 4 mice should yield about 1,5 x10⁷ up to 2 x10⁷ PMNs.
- Bones were opened and bone marrow was flushed out on each side with full-RPMI using a 10ml syringe and a 22G needle into a 50 ml Falcon tube.
- A single cell suspension was made by pipetting bone marrow up and down several times and finally put it through a 70µm cell strainer into a new 50ml Falcon tube.
- The resulting cell suspension was centrifuged for 5min at 300xg, 4°C, and supernatant was removed.
- Red blood cells were lysed by adding 1ml RBC lysis buffer to the cell pellet on ice. After 60sec 20ml full-RPMI was added to cells, stopping the RBC lysis.
- Cells were centrifuged 5min at 300xg, 4°C. Supernatant was removed.

- 2ml layers of each Percoll solution were carefully overlaid with a plastic Pasteur pipette in a 15ml centrifugation tube, starting with the 78% solution down to the 52%.
- Cell pellet was resuspended in 2ml HBSS and cells were layered carefully on top of the Percoll gradient (1 gradient per 4 mice!)
- The gradient was centrifuged at 1500xg and 4°C for 30min using slow start and slow stop.
- A distinct white layer between clear 78% and 69% Percoll layers was collected in a 15ml centrifugation tube (PMNs!)
- The tube was filled up to 15ml with HBSS and cells were centrifuged for 5min at 300xg at 4°C.
- The cell pellet was resuspended in 2-5ml RPMI-full and PMNs were counted in a CASY Cell counter.

10x Phosphate-buffered saline (PBS): 1.4M NaCl pH 7,0
 27mM KCl
 90mM Na₂HPO₄
 15mM KH₂PO₄.

Percoll (Gibco)

Full-RPMI RPMI (with stable glutamine) (PAA)
 + 10% FCS
 + 100 U/ml Penicillin
 + 100µg/ml Streptomycin

2.3 *Candida*-Host Interactions

2.3.1 **Experimental setup for interaction experiments** (for ELISA, signaling analysis and real time PCR)

- One day before *in vitro* host-fungal interaction experiments, BMDMs or mDCs were plated at indicated cell-densities in fresh media and further incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere (incubator).
- For interactions with PMNs, neutrophils were isolated (see 2.2.3), and plated in 24-well plates (Nunc) at indicated cell-densities. Immediately after that, neutrophils were infected with *Candida* or stimulated with other fungal components.
- Fungal cells were prepared for interaction as described in 2.1.3 – 2.1.5 on the day of interaction experiments.

2.3.1.1 *Candida*-BMDM interactions

- Media from BMDM culture dishes was aspirated and replaced with fresh BMDM media (either with or without *Candida*). Typically, interactions of fungi with BMDMs were carried out at an MOI of 2:1 (*Candida*:BMDM). After addition of *Candida* (or only media), dishes were further incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere until collection time.

2.3.1.2 *Candida*-mDC interactions

- Media from mDC culture dishes was aspirated and replaced with fresh mDC media (either with or without *Candida*). Typically, interactions of fungi with mDCs were carried out at an MOI of 2:1 (*Candida*:mDC). After addition of *Candida* (or only media), dishes were further incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere until collection time.

2.3.1.3 *Candida*-PMN interactions

- Shortly after neutrophil isolation, PMNs were supplemented with fresh full-RPMI media (either with or without *Candida*). Typically, interactions of fungi with PMNs were carried out at an MOI of 2:1 (*Candida*:PMN). After addition of *Candida* (or only media), dishes were further incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere until collection time.

2.3.2 **Endpoint dilution killing assay**

- BMDMs or, briefly after isolation, PMNs were plated at 1×10^5 cells per well in a 96 well plate in fresh media and further incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere.
- *Candida albicans* was prepared for interaction as described in 2.1.3.
- *Candida* was diluted in 1:4 or 1:2 serial dilutions (in media) resulting in MOIs of 1:16, 1:64, 1:256, 1:1024 and 1:2048.
- 50µl aliquots of these dilutions were added to wells containing BMDMs respectively PMNs. To block phagocytosis (negative control), 80µM Dynasore (Sigma) were used.
- After addition of *Candida*, dishes were further incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere.
- After 2 days, media was aspirated and cells were fixed with 4% PFA for 10min on ice.
- Cells were washed 2 times with PBS and afterwards stained with Crystal Violet (Sigma) for 20min at room temperature.
- Crystal Violet solution was removed and cells were washed several times with PBS.
- *Candida albicans* plaques stain violet, therefore *Candida* survival and BMDM/PMN killing capacity respectively was assessed. For each dilution, 6 independent assays (wells) were performed.

2.3.3 **Phagocytosis assay**

- BMDMs or mDCs were plated at $2,5 \times 10^5$ cells/well in 2 ml fresh media (6-well plate; Nunc) and further incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere, one day before,
- On the next day, media was aspirated and cells were washed twice with pre-warmed PBS.
- 1ml of fresh media was added and cells were incubated on ice.
- Fluorescently-labelled *Candida* cells (see 2.1.6) were added at an MOI of 2:1 and cells were incubated 45min either on ice (negative control) or at 37°C in a 5% CO₂, 95 % humidity atmosphere.
- Cells were washed three times with PBS and external Alexa Fluor 488 fluorescence indicating not phagocytosed *Candida* was quenched by incubating cells in 0,4% Trypan Blue Solution (Sigma) for 15min at 4°C.
- Cells were washed three times with PBS and finally resuspended in FACS Fix buffer and cooled at 4°C
- Flow cytometric analysis was performed on BD FACS Calibur.

FACS Fix Buffer: PBS
 + 1% (v/v) FCS
 + 4% (v/v) PFA

2.3.4 Apoptosis assay

- BMDMs were plated at 3×10^5 cells per well in a 96 well plate (100 μ l each) in fresh media and further incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere one day before.
- UV-killed *Candida albicans* cells were prepared (see 2.1.5), as well as a Zymosan solution (250 μ g/ml) and a Puromycin solution (Sigma; 50 μ M) as a positive control.
- 50 μ l of either *Candida* cell suspension (MOI 2:1), Zymosan, Puromycin or media alone was added to the BMDMs and cells were incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere for indicated time.
- After interaction, Caspase-Glo 3/7 System (Promega) was used to identify apoptotic cells (for exact measuring procedure see manufacturers guide).

2.3.5 Reactive Oxygen Species (ROS) assay

- BMDMs were plated at $1,2 \times 10^5$ cells per well in a 96-well plate (100 μ l each) in fresh media, and further incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere.
- *Candida* cells were prepared as described in 2.1.3, as well as a Zymosan solution (250 μ g/ml) as a positive control.
- BMDMs were loaded with 0,2mM Luminol and 4 IU/well Horse Radish Peroxidase.
- 50 μ l of either *Candida* (MOI 10:1), Zymosan or media alone was added to BMDMs and plate was placed in pre-warmed Victor Reader (Perkin Elmer).
- Chemiluminescence was measured at 2,5min intervals over a 2hour period (luminescence is expressed as relative luciferase units/minute/1000 cells).

2.3.6 *FACS – staining of immune cells*

- 0,5 x10⁶ cells were incubated with 1µg/10⁶ cells anti CD16/32 antibody (Biolegend, diluted in PBS) for 5 Min at room temperature.
- Afterwards cells were labelled with fluorophor-labelled antibody (1µg/10⁶ cells) for 30 Min at 4°C in the dark.
- Cells were centrifuged at 300xg for 10min at 4°C and washed twice with PBS.
- Cells were resuspended in 500µl FACS Buffer and FACS analysis was performed with BD FACS Calibur (BD Biosciences). For data analysis, FlowJo software (Version 8.8.4) was used.

2.4 Protein Methods

2.4.1 *Protein samples from mammalian cells*

- Fungal-host interaction was performed as described in section 2.3.1.
- After various interaction times, infection was terminated by placing the cell culture dishes on ice and culture media was removed (in case of mDCs were just media was added, culture media was harvested, since it included mDC cells which did not adhere).
- Adherent cells in the culture dish were scraped in 40µl ice-cold protein lysis buffer, collected in a micro-centrifuge tube and centrifuged at 15000xg at 4°C for 10min.
- Supernatants were transferred in fresh tubes containing 15µl of 4x sample buffer plus β-mercaptoethanol (β-ME).
- Samples were heated at 95°C for 5-7min, and afterwards stored at -20°C until further use.

Protein lysis buffer (Frackelton buffer, Kovarik et al, 1998, EMBOJ 17, 3660):

For 10 ml:

Tris 1M pH 7,5	100µl
NaCl 4M	125µl
Triton X-100	100µl
H ₂ O	7575µl
Protease inhibitors (complete no EDTA) 25x (Roche)	40µl
NaPPi 0,3M (RT)	100µl
NaF 1M (-20°C)	50µl
Na vanadate 10mM (-20°C)	10µl
PMSF 100mM (-20°C)	10µl

<u>Sample buffer 4x</u>	1M Tris (pH 6,8)	4ml
	Glycerol	8ml
	SDS	4ml containing 1,6g SDS
	Bromphenol blue	0,4mg
	Water	up to 19,2ml

Add β -ME before use: 96 μ l SBF 4x+ 4 μ l / β -ME

2.4.2 SDS-polyacrylamid gel electrophoresis (SDS-PAGE)

Aliquots (10 μ l) of prepared samples (see 2.4.1) were analysed by SDS-PAGE using standard protocols.

SDS gel:

Resolving gel (10% for 10ml):

Water	4,0ml
30% acrylamide mix	3,3ml
1,5M Tris (pH 8,8)	2,5ml
10% SDS	0,1ml
10% APS	0,1ml
TEMED	0,004ml

Stacking gel (5% for 5ml):

Water	3,4ml
30% acrylamide mix	0,83ml
1,0M Tris (pH 6,8)	0,63ml
10% SDS	0,05ml
10% APS	0,05ml
TEMED	0,005ml

Marker

Page Ruler Prestained Protein Ladder Plus (Fermentas): 5 μ l Marker + 10 μ l sample buffer/ β -ME. 10 μ l were loaded on SDS-PAGE gels.

Running parameters

The gel was run at 20mA constant at 4°C (electrophoresis chamber on ice) until the bromphenol blue front reached the bottom of the gel.

<u>Running buffer 1x</u>	25mM Tris
	192mM glycine
	0,1% SDS

2.4.3 *Western blotting*

I) Semi-dry transfer of proteins

- Blotting sandwich was prepared as follows (semi-dry transfer cell; Biorad “Trans-Blot SD”):
 - On the bottom (=anode electrode) 6 pieces of Whatman-paper soaked with anode-buffer 1
 - 3 pieces of Whatman-paper soaked in anode-buffer 2
 - Nitrocellulose membrane (Immobilin-P, Millipore)
 - SDS-Gel
 - 9 pieces of Whatman-paper soaked in cathode-buffer
- Blotting sandwich was covered with lid (= cathode electrode).
- Proteins were transferred for 1hour at 25V.
- Quality of protein transfer was checked by Ponceau S staining (Fluka Chemika) of the membrane.

<u>Anode buffer 1</u>	0.3M Tris 20% methanol (v/v)
<u>Anode buffer 2</u>	25mM Tris 20% methanol (v/v)
<u>Cathode buffer</u>	25mM Tris 40mM 6-amino-n-hexanoic acid

II) Immunodetection

- To destain membrane from Ponceau S staining, membrane was washed twice for 10min in TBS-T.
- Unspecific binding was blocked by incubating membrane in blocking milk (1x TBS, 5% milk powder (Fixmilch Instant; Maresi) for 1h at room temperature.
- Membrane was washed two times for 5min with TBS-T.
- Primary antibody was diluted in 4% BSA/TBS-T (see Appendix). Membrane was incubated in antibody dilution overnight with continuous shaking at 4°C.
- On the next day membrane was washed three times for 15min with TBS-T.
- Since the Odyssey system (Li-Cor) was used for immunodetection special secondary antibodies were used. All used primary antibodies were raised in rabbit, so IR-Dye800 goat-anti-rabbit antibody (Li-Cor; dilution 1:20000) was used as standard

secondary antibody. Secondary antibodies were diluted in 2% BSA/TBS-T and incubated for 30min at room temperature in the dark.

- Membrane was washed three times for 15min with TBS-T and a fourth time in TBS.
- Membrane was scanned with Odyssey scanner

10x TBS (per litre) 80g NaCl
 2g KCl
 30g Tris, pH 7,4

TBS-T TBS + 0,1% Tween 20

III) Stripping of immunoblots

- Membrane was incubated in pre-warmed stripping buffer (65°C) for 10-15min at room temperature with continuous shaking.
- Stripping buffer was replaced with fresh stripping buffer (65°C) and membrane was incubated for additional 10-15min at room temperature.
- Membrane was washed in TBS-T for 5min
- Finally, membrane was rinsed with PBS and was ready to be used for further immunodetection (2.4.3.II)

Stripping buffer 25mM glycine pH 2.0 + 2% SDS

2.4.4 **Enzyme-linked immunosorbent assay (ELISA)**

For measurement of released cytokines and chemokines in *in vitro* assays or *in vivo* experiments (tissue homogenates, blood,...) ELISAs were performed to quantify protein levels:

I) Plate - ELISA

All ELISAs performed in 96-well plates were purchased from eBioscience. Measuring procedure was performed as described in the manufacturers instructions. Briefly, 96-well plates (Nunc; Maxisorb) were incubated with capturing antibodies (diluted in coating buffer) overnight at 4°C. Next day, capture antibody was aspirated, wells were washed several times with washing buffer and samples (if necessary: diluted in respective culture media) and standards (recombinant; mouse) were added to wells and incubated for 2hours at room temperature with continuous shaking. Samples and standards were removed by aspiration,

wells were washed with washing buffer and detection antibody (diluted in assay diluent) was added to the wells and incubated for 1hour at room temperature with continuous shaking. Detection antibody was aspirated, wells were washed with washing buffer and Avidin-HRP antibody diluted in assay diluent was added to the wells, and incubated for 30min at room temperature with continuous shaking. Avidin-HRP antibody was aspirated, wells were washed with washing buffer and substrate solution was added to the wells and incubated for 15min at room temperature in the dark. Reaction was stopped with stop solution and plate was read at 450nm at Victor Reader (Perkin Elmer).

II) Bead – ELISA

All Bead – ELISAs were purchased from BD Biosciences. Measuring was performed as described in the manufacturers instructions. Briefly, capture beads, PE detection reagent and detection reagent (all for the detection of one particular cytokine, chemokine) were mixed together in tubes. Samples (if necessary: diluted in respective culture media) and standards (recombinant; mouse) were added and incubated for 2hours at room temperature in the dark. Tubes were centrifuged for 10min at 300xg at 4°C and washed two times with washing buffer. Beads were resuspended in washing buffer and analysed on the BD FACS Calibur. Data analysis was performed with BD FCAP Array Software.

2.5 RNA Methods

2.5.1 *RNA extraction from mammalian cells & mouse tissue*

For isolation of total RNA, we used the “SV Total RNA Isolation Kit” (Promega). Isolation was performed as described in the manufacturers instructions. Briefly, cells or tissue homogenates were collected in RNA-Lysis Buffer and diluted with RNA-Dilution Buffer. Samples were heated for 3min at 70°C and afterwards centrifuged at 12000xg for 10min at room temperature. RNA was precipitated by addition of 95% EtOH. Pellet was washed two times with RNA-Washing Buffer and DNA was digested by addition of DNase (15min, room temperature). Resulting RNA pellet was washed two times with RNA-Washing Buffer and RNA was eluted with pre-warmed (65°C) water.

The RNA concentration of RNA was measured with Nanodrop spectrophotometer (Peqlab). Quality of RNA was assessed by running a RNA 6000 Nano Chip (Agilent) on a 2100 Bioanalyzer (Agilent).

2.5.2 *Reverse transcription of RNA samples*

For reverse transcription of RNA, the Reverse Transcription System (Promega) was used.

- An aliquot of RNA (19,6µl, 100ng-1µg RNA) was placed in a Eppendorf tube and heated at 70°C for 10min. After that samples were placed on ice.
- Reverse transcription mix was prepared:

MgCl ₂ 25mM	8µl
Reverse transcription buffer (10x)	4µl
dNTPs 10mM each	4µl
Ribonuclease inhibitor	1µl
Reverse transcriptase	1.4µl (30U)
Oligo (dT) primer	1µl
- RNA sample was mixed with reverse transcription mix and incubated at 42°C for up to 60min.
- Samples were heated at 95°C for 5min and immediately chilled on ice.
- cDNA was diluted with nuclease free water for real-time PCR (1:2 – 1:5 dilution).

2.5.3 *Real-time polymerase chain reaction (RT-PCR)*

- For quantification of gene expression cDNA samples (produced as described in 2.5.1 & 2.5.2) were mixed with the following RT-PCR mix:

KAPA SYBR Green Mastermix (Peqlab)	10µl
Forward Primer (10µM)	0,2µl
Reverse Primer (10µM)	0,2µl
cDNA	5µl
H ₂ O	4,2µl

- Reactions were pipetted in twin.tec real-time PCR plates (Eppendorf), and plates were placed into Realplex⁴ Mastercycler (Eppendorf).
- Following PCR conditions were used:

Denaturation - 95°C	2 Min	40 cycles
Denaturation - 95°C	3 Sec	
Annealing - 62°C	25 Sec	
Elongation & Reading - 72°C	30 Sec	
Melting Curve 10°C	15 Min	

- Each RT-PCR assay data point was measured at least in triplicates.
- For relative quantification of gene expression, data were analyzed according to the $\Delta\Delta C_t$ method. Results are expressed as the -fold expression of the gene of interest versus the expression of a house-keeping gene (always GAPDH) in treated (t) versus untreated (ut) conditions, based on the following equation $R = 2^{-\Delta C_t}$, according to the formula $\Delta\Delta C_t = (\Delta C_{t_{GOI}t} - \Delta C_{t_{GAPDH}t}) - (\Delta C_{t_{GOI}ut} - \Delta C_{t_{GAPDH}ut})$.

2.6 *In Vivo Candida* Infection Studies

2.6.1 *Animals*

For all mouse experiments including isolation of primary cells 7- to 9-week-old male C57BL/6 mice and Tec^{-/-}, Btk^{-/-} as well as Tec^{-/-}Btk^{-/-} mice (Wilfried Ellmeier, Medical University of Vienna, AKH) were used. Mice were maintained in *Candida*-free facilities and *in vivo* infection experiments were carried out in special infection units (Silvia Knapp, Medical University of Vienna, AKH) (BMBWK-66.009/0057-II/10b/2010).

2.6.2 *Systemic Candida albicans* infection – Survival

- For preparation of *Candida albicans* inoculum see 2.1.3
- C57BL/6 wild-type as well as Tec^{-/-} mice were infected with 0,5x10⁵ CFU/mouse intravenously (via tail vein). Control mice received vehicle only (PBS).
- Survival of mice was monitored for up to 35 days
- If mouse reached a human endpoint (criteria for termination of experiment), mouse was sacrificed.

2.6.3 *Systemic Candida albicans* infection

- For preparation of *Candida albicans* see 2.1.3
- C57BL/6 wild-type as well as Btk^{-/-} and Tec^{-/-} mice were infected with 0,5x10⁵ CFU/mouse intravenously (via tail vein). Control mice received PBS.
- On day 7 post infection (p.i.), mice were sacrificed by cervical dislocation and blood (eye vein), as well as organs (kidney, spleen, liver) were collected.

I) Determining fungal burden

- Kidneys of infected mice as well as from control mice were collected, weighted and put in ice cold PBS.
- Afterwards kidneys were homogenized with an Ultra Turrax (IKA); tissue homogenates and serial dilutions of it (1:10, 1:100) were plated on YPD-plates containing ampicillin (120µg/ml), chloramphenicol (40µg/ml) and tetracycline (20µg/ml) (all from Sigma).
- Plates were incubated for 2 days at 30°C and another 24hours at room temperature.
- Colonies of *Candida albicans* were counted (CFU) and calculated back to the total fungal burden per gram (wet weight) kidney as CFU/g.

II) Restimulation of primed splenocytes

- Spleens of infected mice as well as from control mice were collected, weighted and put in ice cold PBS.
- Spleens were homogenized through a 70µm cell strainer (Falcon) and cells were collected in PBS/2% FCS.
- Cells were centrifuged at 300xg for 10min at 4°C
- Pellet was resuspended in Red Cell Lysis Buffer (see 2.2.2) and reaction was stopped after 2min by adding 10ml of PBS/2% FCS.
- Cells were centrifuged at 300xg for 10min at 4°C.
- Pellet was resuspended in full-RPMI media (see 2.2.3) and splenocytes were counted using the CASY cell counter.
- About 2×10^6 splenocytes were distributed into each well of a 24-well plate (Nunc) and cells were incubated at 37°C in a 5% CO₂, 95 % humidity atmosphere.
- UV-killed *Candida albicans* was prepared as described in 2.1.5 and added to splenocytes at an MOI of 1:0,1
- Splenocytes were stimulated for 24hours at 37°C in a 5% CO₂, 95 % humidity atmosphere. Supernatants of cultures as well as RNA were collected and used for ELISA measurements and RT-PCR.

III) Characterization of splenocytes

- Splenocytes were isolated as described in 2.6.2.II.
- 1×10^6 splenocytes were distributed in FACS tubes and 1ml of FACS buffer was added. Cells were centrifuged at 300xg for 10min at 4°C.
- Cells were resuspended in 50µl FACS buffer containin 1µl anti CD16/32 antibody (Biologend).
- Cells were incubated for 30min at 4°C in the dark with following antibody mixes (in 50µl FACS buffer):

Myeloid Panel

Epitop	Fluorophor	Volume (µl)	Cells
CD45	APC-Cy7	1	Immune
CD11b	FITC	1	Mph
F4/80	APC	1	Mph
CD11c	PerCP-Cy5.5	1	DC
PDCA1	PE	0.8	DC
Ly6c	V450	1	DC
B220	V500	1	B cell
MHCII	PE-Cy7	1	APC

Lymphoid Panel

CD45	APC-Cy7	1	Immune
CD3	FITC	1	T cell
CD4	APC	1	T cell
CD8	V450	1	T cell
CD25	PE-Cy7	1	T cell
NK1.1	PE	1	NK cell
CD19	PerCP-Cy5.5	1	NK / NKT cell
B220	V500	1	B cell

- 1ml FACS buffer was added and cells were centrifuged at 300xg for 10min at 4°C
- Cells were resuspended in 500µl and cells were analysed on FACS Aria (BD). For data analysis FlowJo software (Version 8.8.4) was used.

IV) Isolation and restimulation of T cells

- Splenocytes were isolated as described in 2.6.2.II.
- Cells were resuspended in 500µl antibody mix/spleen (see below, all Biotin-coupled) and incubated for 40min on ice.

	Final concentration (µg/ml)	Company
Gr-1 (granulocytes/neutrophils)	4	BD Biosciences
B220 (B cells)	4	BD Biosciences
Ter119 (erythrocytes)	1	BD Biosciences
NK (natural killer)	1	BD Biosciences
CD11c (dendritic cells)	1	BD Biosciences
CD11b (macrophages)	1	BD Biosciences
CD8 (T cells)	2	BD Biosciences

- 10ml PBS/2% FCS buffer were added and cells were centrifuged for 5min at 300xg at 4°C.
- 200µl of Streptavidin-coupled beads (BD Biosciences) were added per spleen and incubated for 20min on ice.
- 400µl PBS/2% FCS buffer were added and cells were put on a magnet. Supernatant was collected and beads were washed twice with 1ml PBS/2% FCS buffer.
- All supernatants were collected and put on a magnet. Resulting final supernatant (contained only CD4⁺ T cells) was centrifuged at 300xg for 5min at 4°C.
- Cell pellet was resuspended in 1ml full-RPMI media / spleen and cells were counted using CASY-Cellcounter and FACS characterized (2.3.6).
- For restimulation, 1x10⁶ CD4⁺ T cells were put in 6-well plates (Nunc) and stimulated with 1µg/ml anti-CD3 ϵ and 3µg/ml anti-CD28 (both Biologend) into RPMI media. Cells were stimulated for 4hours at 37°C in a 5% CO₂, 95 % humidity atmosphere in the presence of GolgiPlug (BD Biosciences).

For intracellular staining, “Intracellular Staining Kit” (BD Biosciences) was used. Staining was performed as described in the manufacturers instructions.

Briefly, cells were centrifuged at 300xg for 10min at 4 °C and resuspended in 100µl Staining Buffer. To block Fc-receptors, 1µg/10⁶ cells anti CD16/32 antibody (Biolegend) was used and cells were incubated at 4°C for 15min. Cells were centrifuged and resuspended in 100µl Fixation/Permeabilization Buffer. Permeabilized cells were centrifuged and resuspended in 50µl Perm/Wash Buffer containing anti PE-Foxp3 (1µg/10⁶ cells) antibody (Biolegend) and incubated for 30min at 4°C. Cells were washed twice in Perm/Wash Buffer, and resuspended in 250µl Staining Buffer prior to FACS analysis. FACS analysis was performed with the BD FACS Calibur (BD Biosciences). For data analysis, FlowJo software (Version 8.8.4) was used.

V) Dendritic cell (mDC) / T cell Co-culture

- Dendritic cells were differentiated as described in 2.2.2. One day before interaction, 1×10^5 mDCs/well (96-well plate, Nunc) were plated in RPMI media and stimulated with UV-killed *Candida albicans* (see 2.1.5) at an MOI of 1:1.
- T cells were isolated as described in 2.6.2.IV and $0,5 \times 10^6$ Tcells were added to mDC wells (MOI of 5:1).
- Co-culture was incubated for 72hours at 37°C in a 5% CO₂, 95 % humidity atmosphere.
- Supernatants of culture were collected and cytokines were measured using ELISA-measurments (2.4.4)

VI) Urea in blood (BUN)

Blood of infected and uninfected mice was collected (trans-orbital), centrifuged at 4000xg for 15min at 4 °C. Sera was taken off. Levels of blood urea nitrogen (BUN) were quantified by In Vitro Diagnostics (1030 Vienna, Austria).

2.7 Statistics

All statistical analyses were performed using Prism 5.0a (GraphPad Software, Inc.). P values were calculated by Student's t test with unpaired, two-tailed distribution.

P-values for survival-experiments were calculated by the log-rank (Mantel-Cox) test. P values such * p-value < 0,05 ; ** p-value < 0,01 ; *** p-value < 0,001 were considered significant.

3 RESULTS

3.1 *In Vivo* Role of Tec Family Kinases in anti-fungal Response

The role of Tec family kinases during microbial infections is only poorly understood. Only a few studies reported involvement of Tec family kinases in selected microfilarial, bacterial or viral infections, but none of these studies addressed the *in vivo* relevance of Tec family kinases in fungal infections. Thus, Tec^{-/-} as well as Btk^{-/-} mice were used as models to study the role of Tec family kinases during infections with *C. albicans*.

3.1.1 *Survival of mice infected with C. albicans*

To assess the *in vivo* relevance of Tec family kinases, Tec^{-/-} and WT control mice were infected intravenously with 0,5 x 10⁵ CFU/mouse (control group: PBS). As previously described, mice with systemic candidiasis die of progressive sepsis, with the kidneys being the most heavily affected organ (Spellberg B. et al, 2005). Hence, the health-status of mice was monitored at least twice a day.

Strikingly, Tec^{-/-} mice showed highly significant improved survival and thus resistance when compared to their wild-type littermates (Figure 3.1 A). After 5 weeks, survival experiments were terminated and Tec^{-/-} mice were sacrificed. Kidneys of Tec^{-/-} mice were collected and fungal burdens were determined. Surviving Tec^{-/-} mice revealed a quite inhomogenous picture when looking at the kidney CFUs of *C. albicans* in those mice. While there were mice, which completely cleared the infection, there were still other animals, which show presence of *Candida* in their kidneys (B).

Notably, kidneys of all surviving mice revealed very diverse appearances. While one of the kidneys showed normal architecture, colour and no signs of organ damage, the other kidney was swollen, exhibited a yellowish colour and was filled with pus. Additionally, blood samples of WT and Tec^{-/-} mice were taken and serum-levels of BUN (blood urea nitrogen) were assessed (C), since increase in BUN-levels have been associated with loss of kidney function in *C. albicans* infections (Spellberg B. et al, 2005). An increase in BUN levels of WT and Tec^{-/-} mice after infection with *C. albicans* was detectable. However, BUN-levels of Tec^{-/-} mice after 7 days p.i. were significantly lower when compared to their wild-type littermates, indicating that Tec^{-/-} mice show decreased signs of kidney damage under these infection conditions.

Surviving *Tec*^{-/-} mice did not exhibit elevated BUN levels, although, they showed clear morphological signs of kidney damage as mentioned earlier.

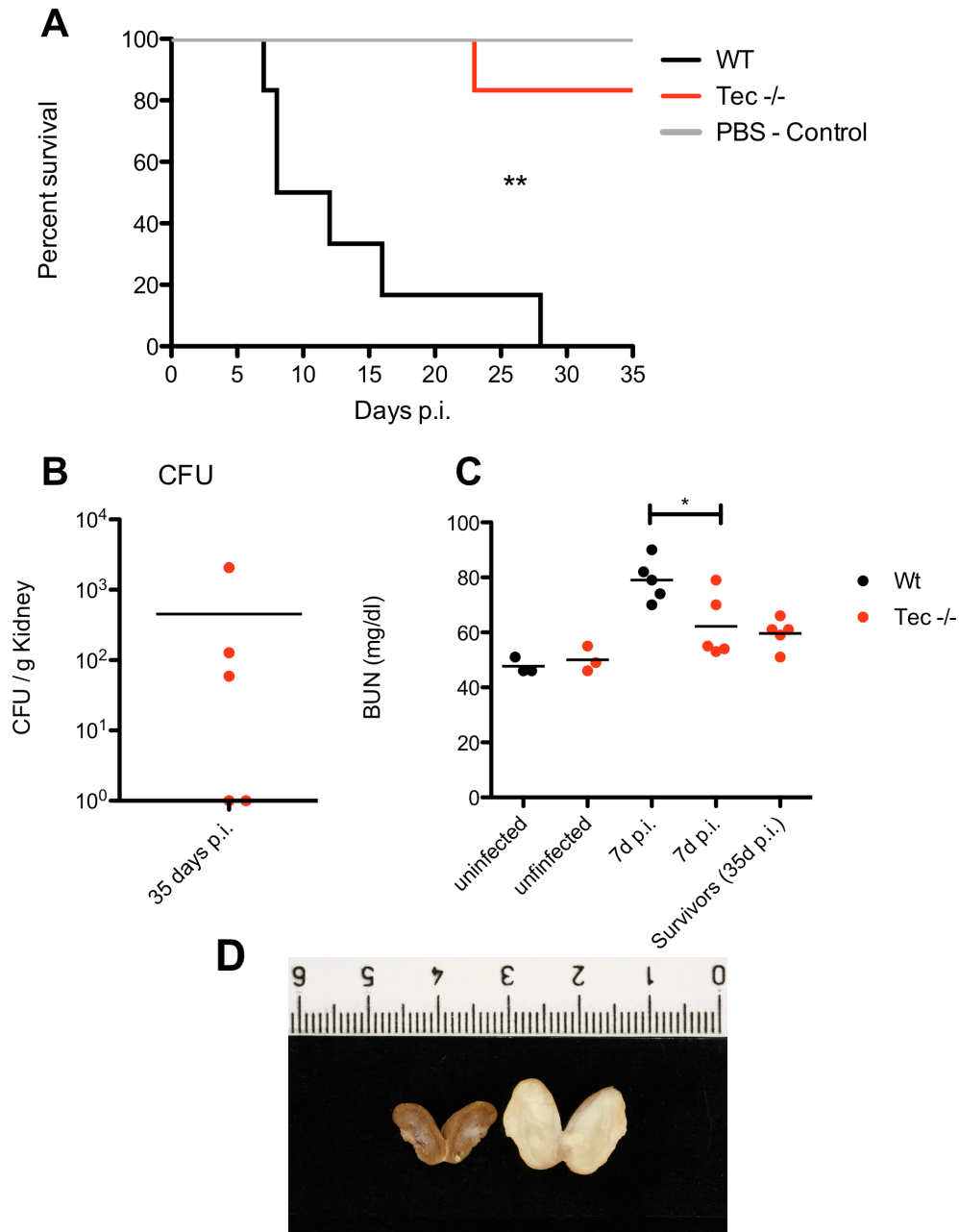


Figure 3.1: Survival of mice challenged with *C. albicans*

WT and *Tec*^{-/-} mice (all C57BL/6) were challenged with 0.5×10^5 live *C. albicans* i.v. per mouse. Survival of mice was followed up for 35 days, mice were monitored at least two times a day (A; n=6). Kidneys of surviving *Tec*^{-/-} mice were collected, homogenized and plated on YPD-plates containing Tet, Amp and Chl. Plates were incubated for 2 days at 30°C and colonies of *C. albicans* were counted and calculated back to the total fungal burden per gram kidney (B; n=5). Blood of mice was collected and sera-levels of BUN were measured (C; n=3-5). Kidney pathology of moribund WT mouse (D).

3.1.2 Mice challenged with *C. albicans*

To test if the increased survival is Tec-specific or if other Tec kinase family members are implicated in the immune response to *C. albicans*, infection experiments were also performed with Btk^{-/-} mice. Therefore, Tec^{-/-} and Btk^{-/-} mice were challenged with *C. albicans* as described above. Mice were infected intravenously via tail vein with a dose of $0,5 \times 10^5$ CFU/mouse. Mice were sacrificed 7 days post-infection (p.i.) and samples collected (blood, kidneys, spleen). Mice challenged with *C. albicans* lost up to 15% of their body weight in the first 7 days of infection (Figure 3.2). Notably, Btk^{-/-} mice suffer most upon infection, since these mice showed a higher weight loss (Figure 3.2 A), suggesting a severe condition. Additionally, one Btk^{-/-} mouse died in the first 7 days of infection, unlike WT or Tec^{-/-} mice. Furthermore, spleen weight of infected mice was also measured. Interestingly, Btk^{-/-} mice showed a highly significant reduced spleen weight after 7 days post infection (Figure 3.2 B) when compared to spleens of wild-type and Tec^{-/-} mice. Thus, that Btk^{-/-} mice are likely to have a severe problem in mounting an adequate adaptive immune response and mice may already be “overburdened” with this particular challenge-dose of *C. albicans*.

Kidneys of infected mice were also collected, homogenized and fungal burdens were assessed by quantifying colony forming units (CFUs) of *C. albicans* (Figure 3.2 C). Strikingly, Tec kinase-deficient mice showed highly significant increased fungal burdens in their kidneys than kidneys from WT mice, There were no differences in fungal burdens between Tec^{-/-} and Btk^{-/-} mice were observed, at least in kidneys. Next, sera-levels of different cytokines and chemokines were determined. Blood of the mice was collected and ELISAs were performed. However, there were no significant differences in serum-levels of IL-6 or the chemokine KC between wild-type and Tec kinase-deficient mice after 7 days of challenge with *C. albicans* (Figure 3.3)

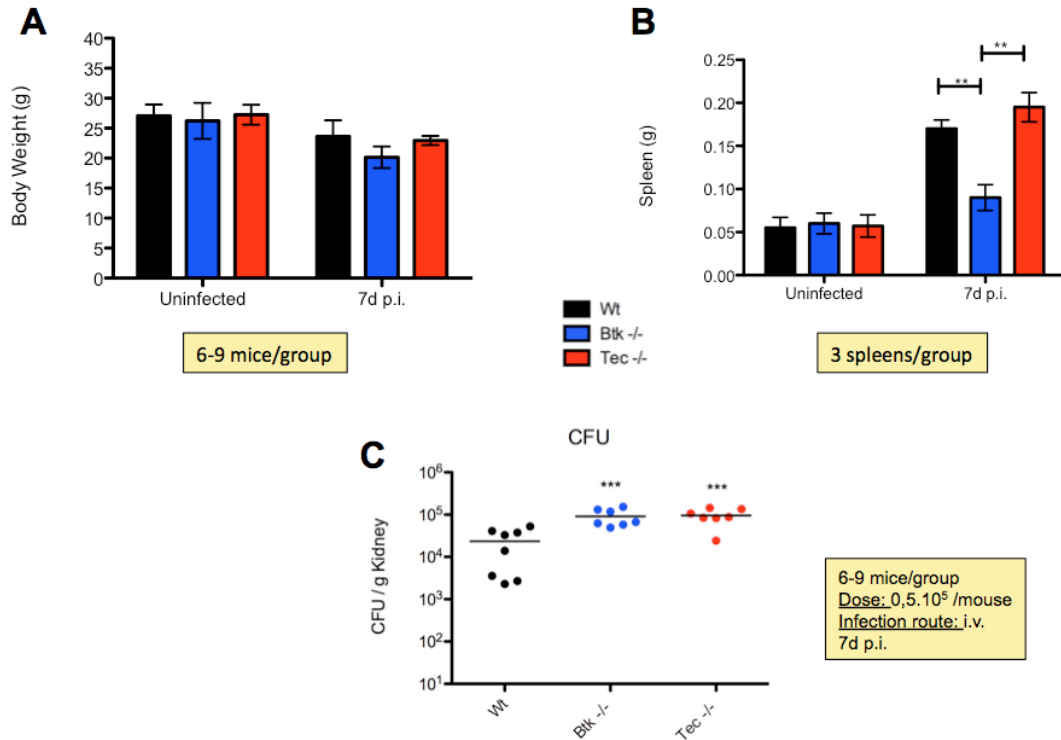


Figure 3.2: **Mice challenged with *C. albicans***

WT, Btk^{-/-} and Tec^{-/-} mice (all C57BL/6) were challenged with a fungal dose of $0,5 \times 10^5$ live *C. albicans* i.v. per mouse. After 7 days, body weight of mice (A; n=6-9) and spleen weight (B; n=3) were assessed. Kidneys were taken, homogenized and plated on YPD-plates containing Tet, Amp and Chl. Plates were incubated for 2 days at 30°C and grown colonies of *C. albicans* were counted and calculated back to the total fungal burden per gram kidney (C; n=7-8).

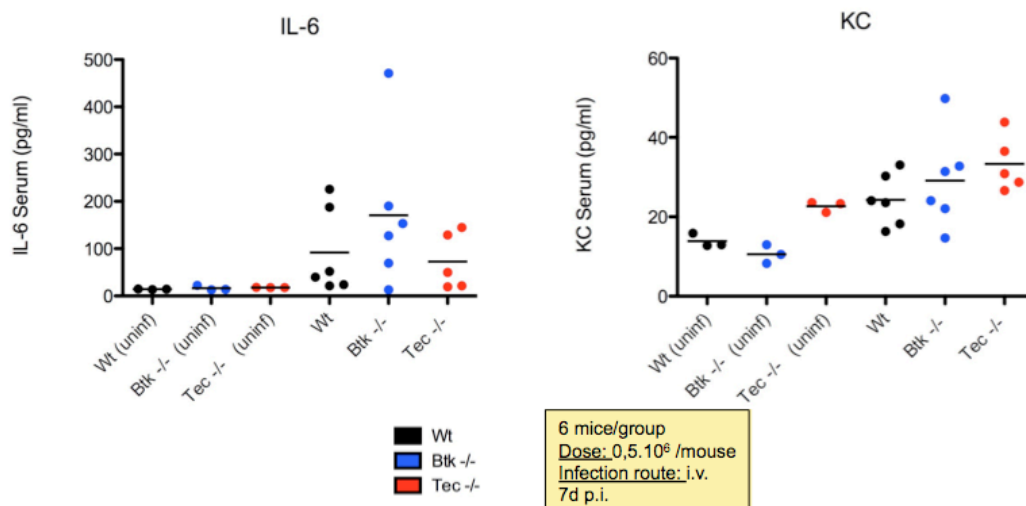


Figure 3.3: **Mice challenged with *C. albicans***

WT, Btk^{-/-} and Tec^{-/-} mice (all C57BL/6) were challenged for 7 days with $0,5 \times 10^5$ live *C. albicans* i.v. per mouse. After 7 days, blood of mice was taken and serum-levels of IL-6 and KC were measured using ELISA. n=3-6

3.1.3 **Restimulation of splenocyte cultures**

To test for adaptive immune responses, which were primed *in vivo*, restimulations of splenocyte-cultures were performed. For this purpose, spleens of infected (dose: $0,5 \times 10^5$ CFU/mouse; i.v.) mice were taken and homogenized. The resulting crude cell suspension was restimulated with heat-killed *C. albicans* and released cytokines (signature cytokines for each Th-lineage) were quantified by ELISA, using IFN- γ for Th-1 cells, IL-4 for Th-2, IL-17 for Th-17 and IL-10 for regulatory T cell (Figure 3.4). Compared to splenocyte cultures of WT mice, both *Tec*^{-/-} and *Btk*^{-/-} splenocytes showed significantly higher IFN- γ release upon *Candida*-restimulation, indicating an augmented Th-1 response (Figure 3.4 D).

For IL-4, no differences were observed for any of the Tec kinase-deficient splenocyte-cultures, indicating no major changes in the Th-2 driven immune answer to *C. albicans* (Figure 3.4 A).

For Th-17, the picture was not clear. *Btk*^{-/-} splenocytes revealed no changes in IL-17 release upon restimulation. By contrast, *Tec*^{-/-} splenocytes showed higher IL-17 production, albeit, no significance level was reached (Figure 3.4 B).

Strikingly however, *Tec*^{-/-} splenocytes released highly significant increased levels of IL-10. This seems to be a Tec-specific feature, since *Btk*^{-/-} splenocytes produced same amount of IL-10 as WT splenocytes upon restimulation (Figure 3.4 C). Hence, increased levels of IL-10 in *Tec*^{-/-} splenocyte-cultures could implicate a polarisation towards regulatory T cells occurring in infected *Tec*^{-/-} mice.

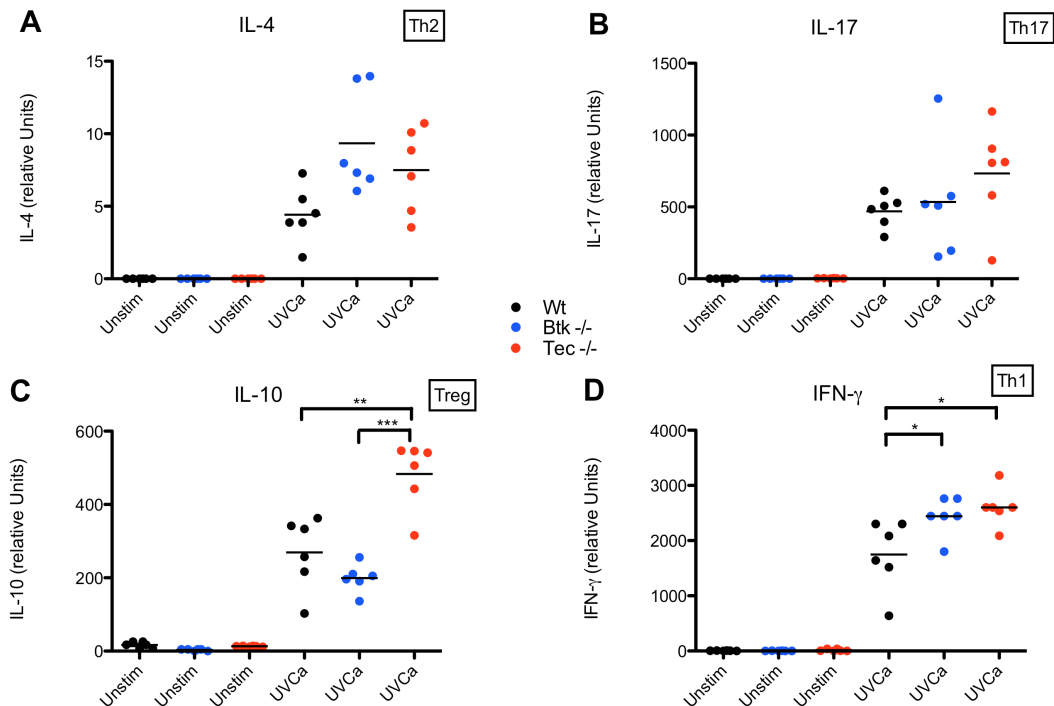


Figure 3.4: **Restimulation of splenocyte-cultures with heat-killed *C. albicans***

WT, Btk^{-/-} and Tec^{-/-} mice (all C57BL/6) were challenged with 0.5×10^5 live *C. albicans* i.v. per mouse. After 7 days, spleens were taken, homogenized and resulting cell suspension was restimulated with heat-killed *C. albicans* (MOI 1:0,1) for 24 hours. Supernatants were collected and released cytokines were measured with ELISA. n=6

Hence, these cytokine data implied a rather strong induction of a tolerogenic immune answer in Tec^{-/-} splenocytes, which may explain the observed resistance phenotype in survival experiments.

To further analyze this observation, RNA of splenocyte-cultures was collected and real-time PCR analyses were performed.

The enzyme indoleamine 2,3-dioxygenase (IDO) has been reported to mediate tolerogenic responses in *C. albicans* infections (Romani L. and Puccetti P., 2006). IDO is able to catabolize tryptophan, thereby influences T cell responses by strongly inducing IL-10 expression followed by massive differentiation of regulatory T cells. However, none of the different splenocyte-cultures (WT, Btk^{-/-}, Tec^{-/-}) showed an induction of IDO-expression, rather repression was observed, since values of stimulated samples are lower than 1 (unstimulated sample) (data not shown).

Recently, another tolerogenic pathway has been described operating in dendritic cells to induce tolerogenic immune answers (Pulendram B. et al, 2010). The stimulation of dendritic cells via TLR2/TLR6 leads to increased expression of retinaldehyde dehydrogenase 2 (RALDH2) and IL-10. RALDH2 is able to metabolize vitamin A into retinoic acid, which in turn signals back in an autocrine manner to induce production of suppressor of cytokine signaling 3 (SOCS3). Hence, this provides an environment, favouring emergence of Foxp3-positive regulatory T cells and Tr-1 cells (Figure 3.5).

Indeed, restimulated *Tec*^{-/-} splenocytes show upregulation of different components of this signaling-pathway when compared to wild-type controls. RALDH2 (Figure 3.5 A), SOCS3 (B) and TGF- β (C) show significant transcriptional upregulation in stimulated *Tec*^{-/-} samples. Furthermore, Foxp3 (D) was also upregulated. By contrast, *Btk*^{-/-} splenocytes failed to show any upregulation in comparison to wild-type controls for either one of the signaling components. Thus, this data suggest a Tec-specific function upon *Candida* challenge. Moreover, these data also confirm a role of the Treg lineage in tolerance again.

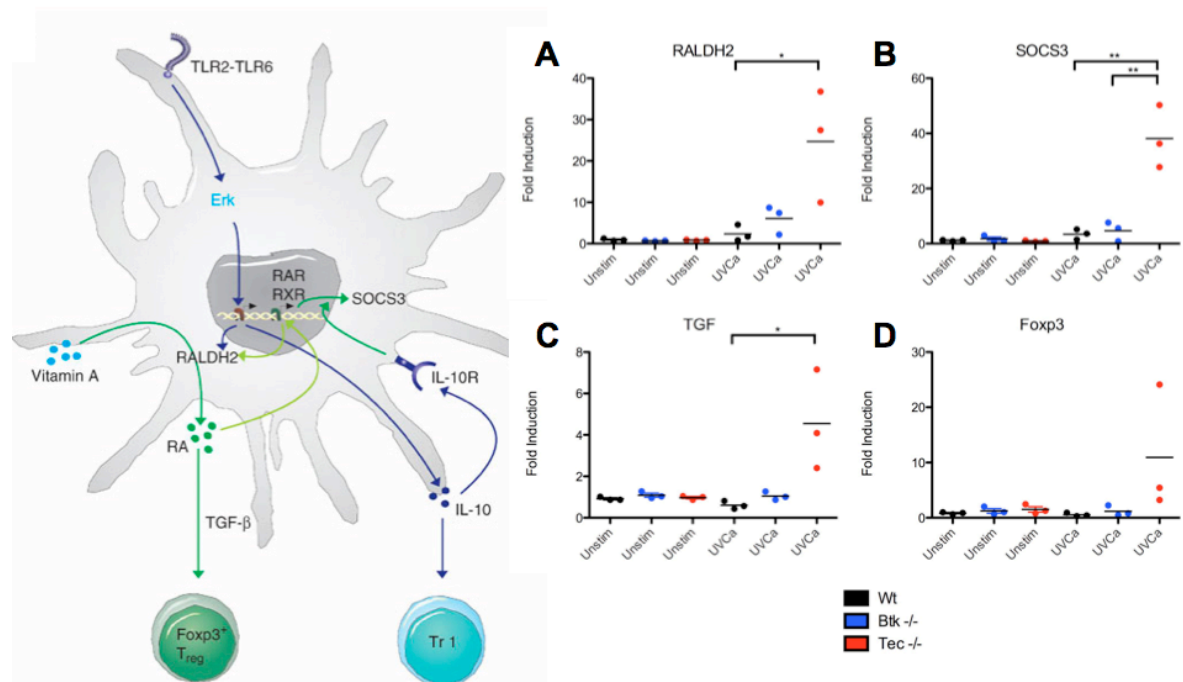


Figure 3.5: Tolerogenic pathway in *Candida*-challenged splenocyte-cultures

WT, *Btk*^{-/-} and *Tec*^{-/-} mice (all C57BL/6) were challenged with 0.5×10^5 live *C. albicans* i.v. per mouse. After 7 days, spleens were taken, homogenized and resulting cell suspension was restimulated with heat-killed *C. albicans* (MOI 1:0,1) for 24 hours. RNA samples were taken, reversely transcribed and real-time PCR was performed (housekeeping gene: GAPDH; for primer sequences see Appendix). Expression of RALDH2 (A), SOCS3 (B), TGF- β (C) and Foxp3 (D) was assessed. n=3. Cartoon was taken from Pulandram B. et al, 2010

Since SOCS proteins have been implicated in tolerogenic immune answers (Dimitriou ID. et al, 2008), we further analyzed splenocyte-cultures for expression of all known SOCS proteins, using a real-time PCR approach (Figure 3.6)

Interestingly, *Tec*^{-/-} splenocytes exhibited elevated expression of SOCS1 (Figure 3.6 A), SOCS2 (C), SOCS3 (D) and SOCS4 (F), while CIS(H) (B) and SOCS6 (E) did not show any upregulation when compared to wild-type splenocytes. Again, *Btk*^{-/-} splenocytes did not display any increase in expression levels for either of the different SOCS proteins compared to WT splenocytes, indicating a *Tec*-specific feature.

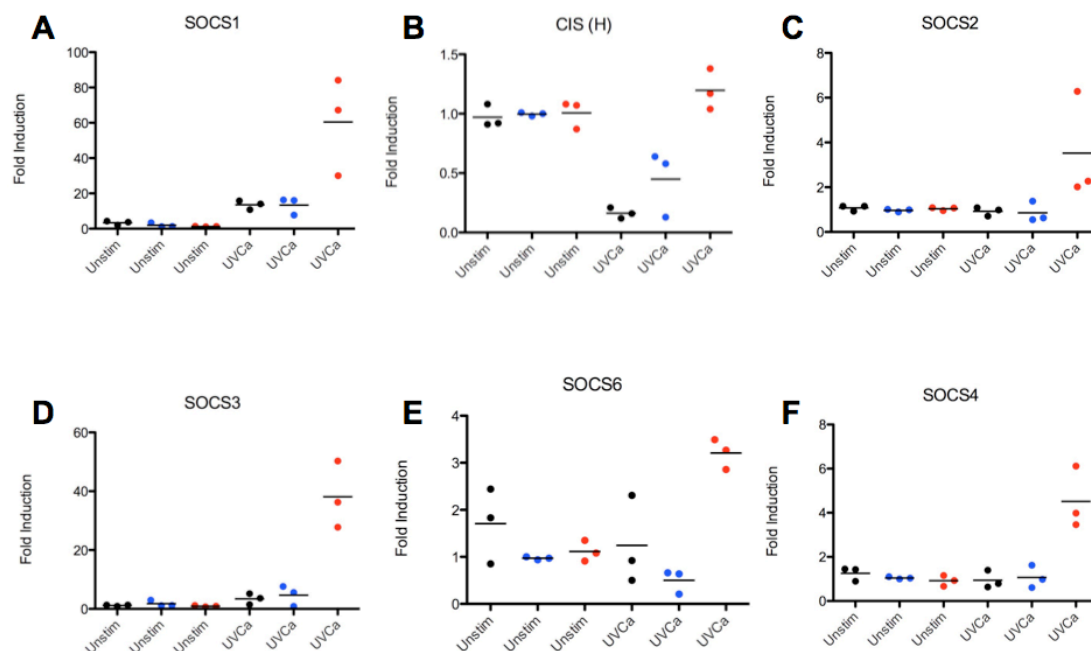


Figure 3.6: **SOCS-expression in *Candida*-challenged splenocyte-cultures**

WT, *Btk*^{-/-} and *Tec*^{-/-} mice (all C57BL/6) were challenged with 0.5×10^5 live *C. albicans* i.v. per mouse. After 7 days, spleens were taken, homogenized and resulting cell suspension was restimulated with heat-killed *C. albicans* (MOI 1:0,1) for 24 hours. RNA samples were taken, reversely transcribed and real-time PCR was performed (housekeeping gene: GAPDH; for primer sequences see Appendix). Expression of SOCS1 (A), CIS(H) (B), SOCS2 (C), SOCS3 (D), SOCS6 (E) and SOCS4 (F) was assessed. n=3

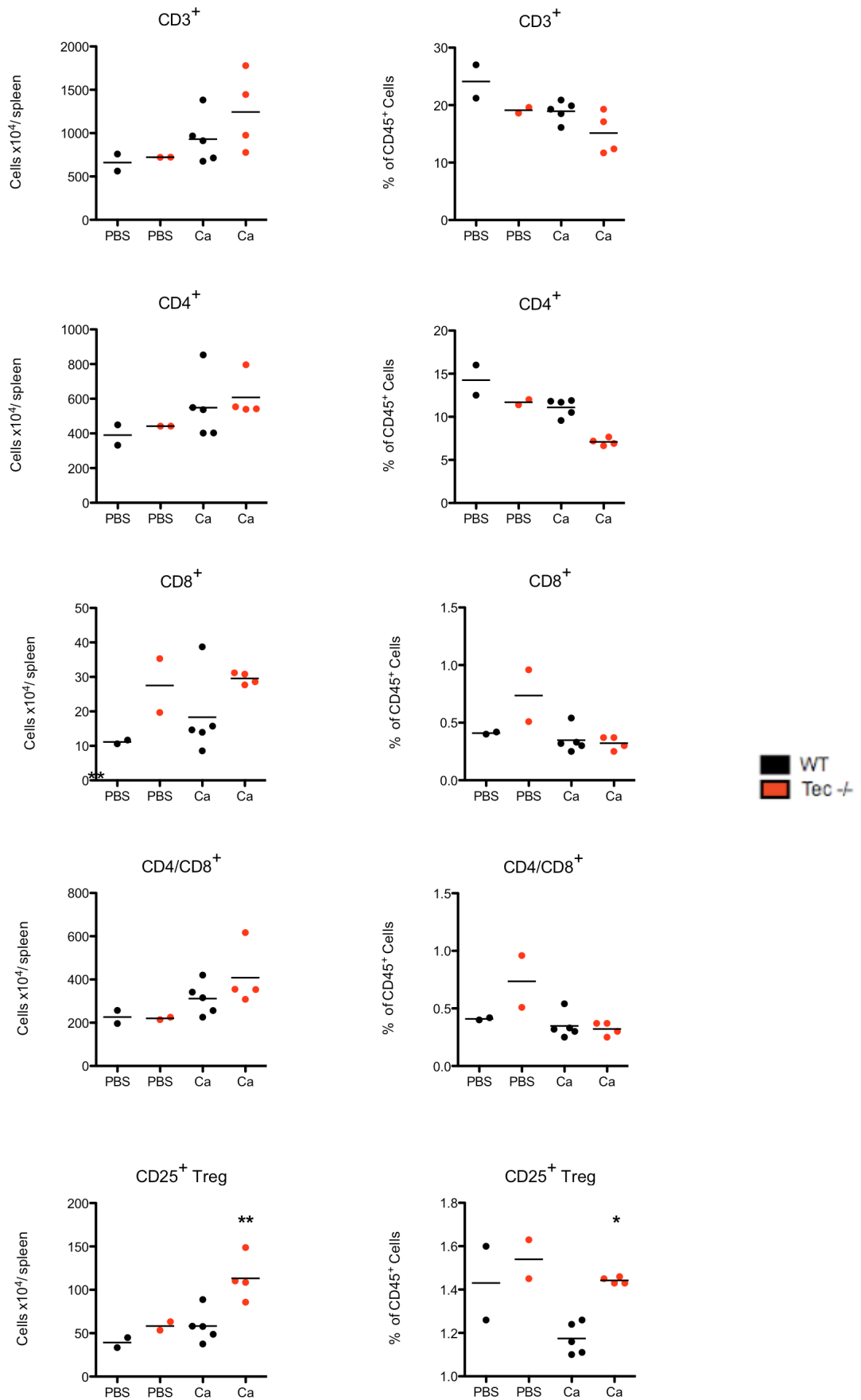
Since $Tec^{-/-}$ mice revealed improved survival, and because restimulation experiments of $Tec^{-/-}$ splenocyte-cultures showed significantly higher IL-10 release, elevated IFN- γ production and a tendency to increased IL-17, we were interested in the detailed composition of WT respectively $Tec^{-/-}$ splenocyte cultures. Hence, overrepresentation of a certain cell type (or several cell types) could provide clues about the nature of immune cells mediating the changes in cytokine profile upon *Candida* stimulation. We therefore collected spleens of uninfected and infected mice (7 day p.i.; dose: $0,5 \times 10^5$ CFU/mouse; i.v.), and homogenized them. The resulting cell suspension was stained with FACS antibodies and FACS analysis was performed to determine the amount of various lymphoid as well as of myeloid cells, based on distinct markers (see Appendix) (Figure 3.7 and Figure 3.8).

For $CD3^+$ T cells, an increase in total cell number and a decrease based on percentage of present leukocytes ($CD45^+$) upon *Candida* challenge, could be detected. No significant changes between WT and $Tec^{-/-}$ mice were assessed. The same pattern was also observed for $CD4^+$, $CD8^+$ and $CD4/CD8^+$ T cells (Figure 3.7 A). Strikingly, splenocytes from infected $Tec^{-/-}$ mice revealed significantly elevated levels of $CD25^+$ regulatory T cells (total as well as ratio of $CD45^+$ cells). This observation confirms our results, since regulatory T cells are the major drivers in tolerogenic immune responses.

B cell levels increased upon infection with *C. albicans*. The total number of B cells from infected $Tec^{-/-}$ mice was significantly higher than in WT splenocytes. Notably, there was no significant change in amount of B cells based on all $CD45^+$ immune cells (Figure 3.7 B).

Finally, total numbers of Natural Killer cells (NK cells) remained similar upon infection with *C. albicans*, although a decrease of NK cells relative to $CD45^+$ immune cells could be assessed. Natural Killer T cells (NKT cells) exhibited an increase in total number as well as an increase among all immune cells during challenge with *C. albicans*, showing no significant changes between WT and $Tec^{-/-}$ mice (Figure 3.7 C).

A



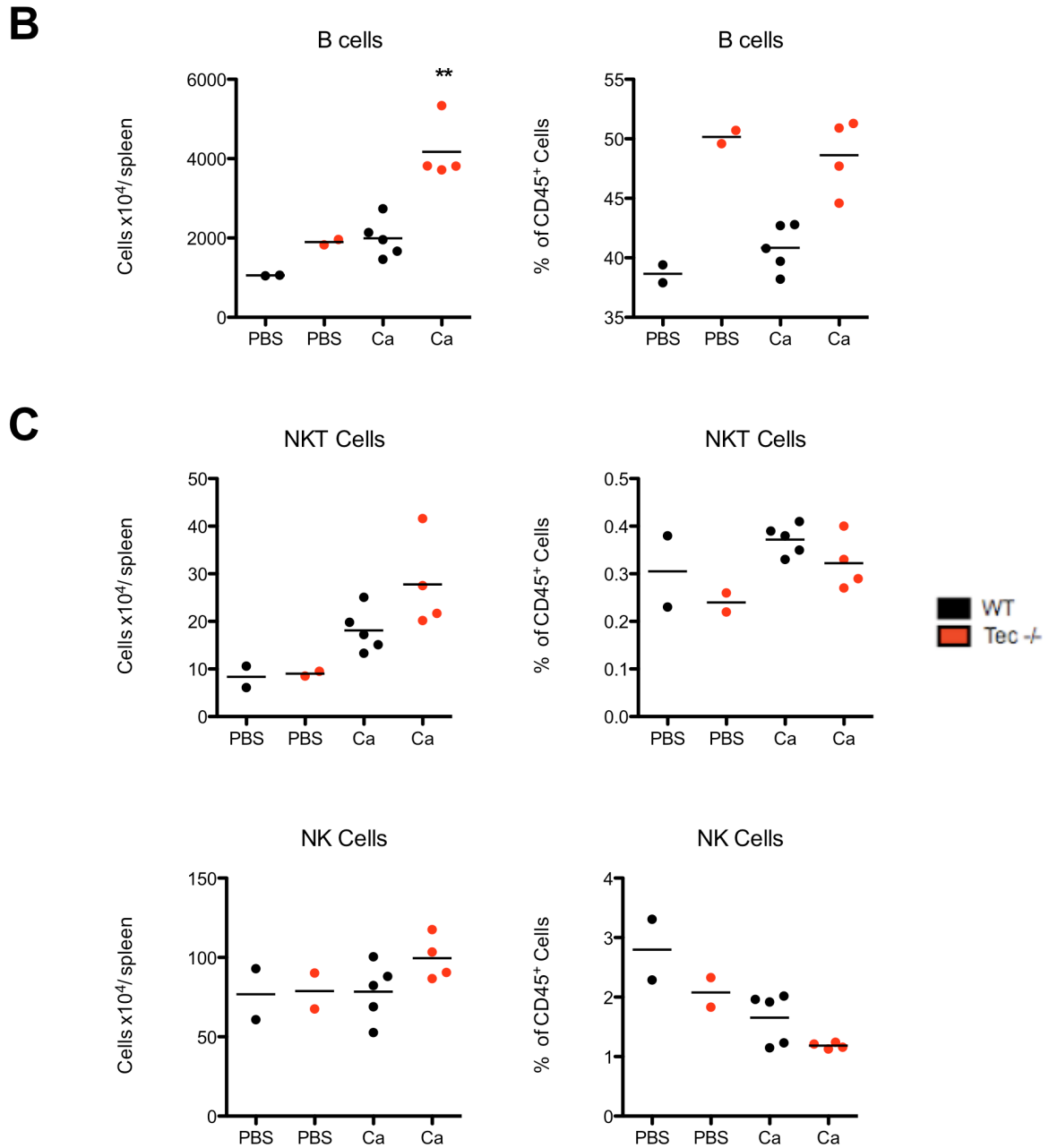
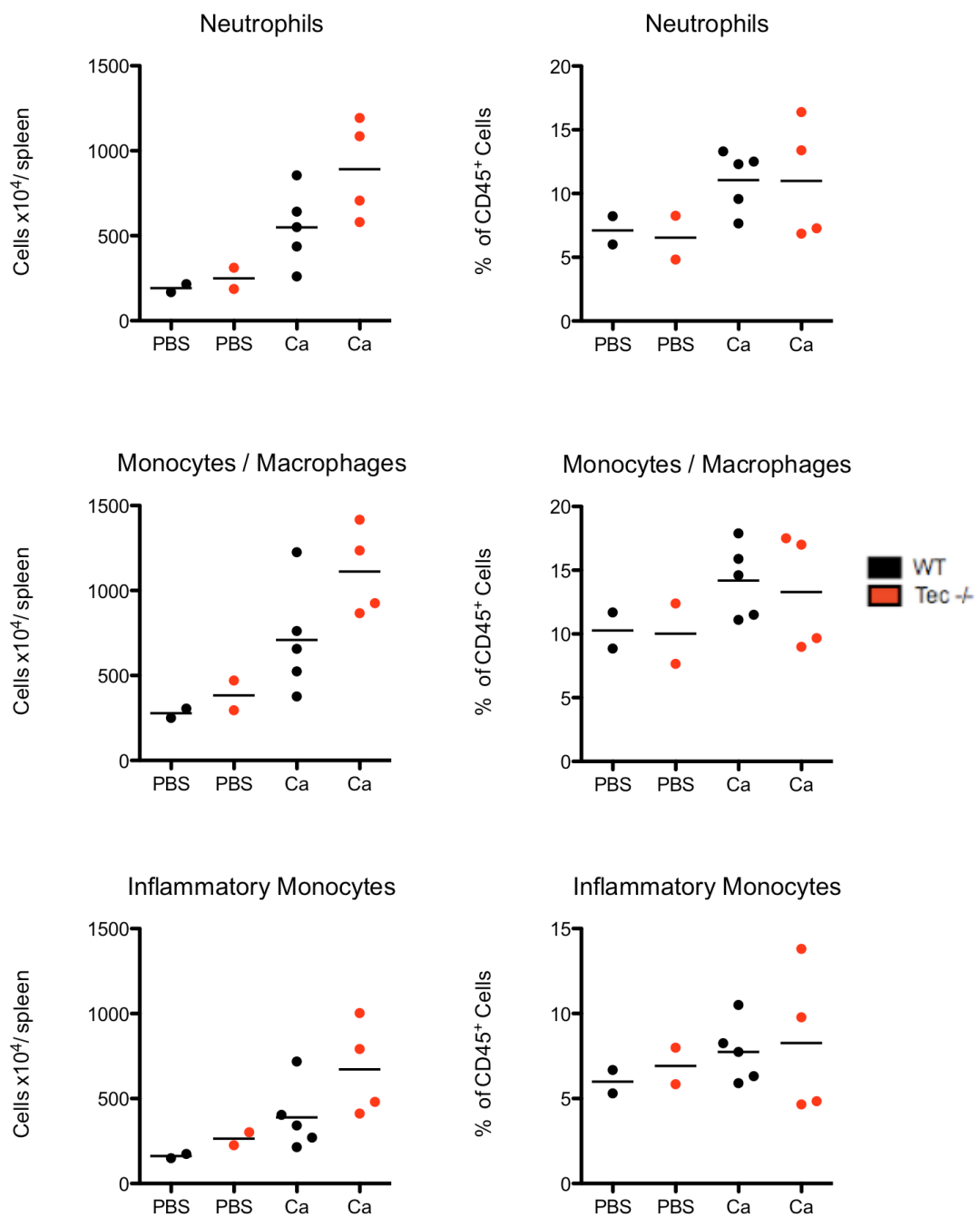


Figure 3.7: Spleen-composition of mice infected with *Candida albicans* – lymphoid panel

Wt Tec^{-/-} mice (all C57BL/6) were challenged with $0,5 \times 10^5$ live *Candida albicans* i.v. per mouse. After 7 days, spleens were taken, homogenized and resulting cell suspension was stained with FACS-antibodies. Stained cells were analyzed with BD FACS Aria and populations were assessed based on cell markers (see Appendix). CD45⁺ immune cells were gated. Levels of T cells (CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, CD3⁺CD4/CD8⁺, CD3⁺CD4⁺CD25⁺) (A), B cells (CD19⁺B220⁺) (B) and NK cells (NK1.1⁺CD3⁺) and NKT cells (NK1.1⁺CD3⁺) (C) were assessed. n=2-5

When looking at cells of the myeloid lineages, the homogeneity of different cell populations between mice of the same genotype changed during the course of infection (Figure 3.8). Differences in the amount of various myeloid populations were especially observed in samples from *Tec*^{-/-} mice. Nevertheless, changes in cellular composition of the spleen in mice infected with *C. albicans* was observed. Monocytes/macrophages, as well as neutrophils, exhibited increased cell numbers in spleens upon challenge with *C. albicans*, albeit with no differences in these populations between WT and *Tec*^{-/-} mice. Likewise, inflammatory monocytes and inflammatory dendritic cells in spleens of infected mice, showed no significant alterations between WT and *Tec*^{-/-} samples. However, *Tec*^{-/-} mice exhibited elevated levels of splenic and plasmacytoid dendritic cells (total cell number) in response to infection with *C. albicans* when compared to their wild-type littermates. While levels of plasmacytoid dendritic cells did not reach levels of significance (n= 4-5), splenic dendritic cells showed highly significant elevated cell numbers. Noteworthy, when looking at the percentages of CD45⁺ cells (leukocytes) rather than on total cell numbers, splenic and plasmacytoid dendritic cells seem to be the only myeloid cell populations which failed to increase upon challenge with *C. albicans*.

Results



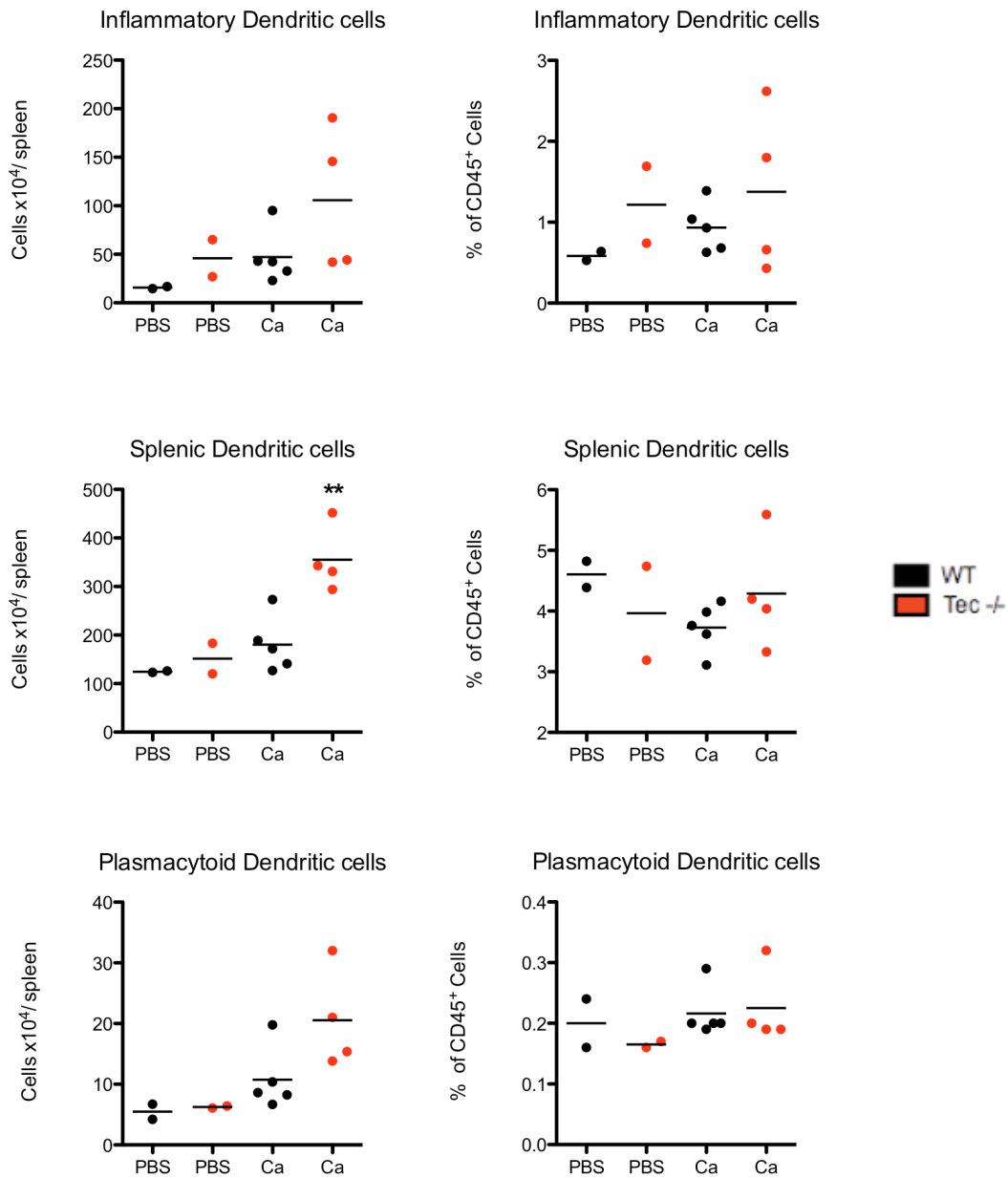


Figure 3.8: **Spleen-composition of mice infected with *C. albicans* – myeloid panel**

WT and *Tec*^{-/-} mice (all C57BL/6) were challenged with 0.5×10^5 live *C. albicans* i.v. per mouse. After 7 days, spleens were taken, homogenized and resulting cell suspension was stained with FACS-antibodies. Stained cells were analyzed with BD FACS Aria and populations were assessed based on cell markers (see Appendix). CD45⁺ immune cells were gated. Levels of neutrophils (CD11b⁺ Ly6c⁺ CD11c⁻ F4/80⁻), monocytes/macrophages (CD11b⁺ Ly6c^{+/+} CD11c⁻ F4/80⁺), inflammatory monocytes (CD11b⁺ Ly6c⁺ CD11c⁻ F4/80⁺), inflammatory DCs (CD11b⁺ Ly6c⁺ CD11c⁺ F4/80⁺), splenic DCs (CD11b^{+/+} Ly6c⁻ CD11c⁺ F4/80⁻), and plasmacytoid DCs (CD11b⁻ Ly6c⁺ CD11c⁻ F4/80⁺PDCA1⁺) were assessed. n= 2-5

3.2 Functional Characterization of Tec Family Kinase-deficient Immune Cells

To determine the underlying molecular mechanisms mediating improved survival of *Candida*-challenged Tec^{-/-} mice, altered fungal burdens and changes in the adaptive immune response *in vitro* cell culture models were used. To investigate if Tec kinases show cell-type specific, non-redundant functions, bone marrow of Tec^{-/-} as well as Btk^{-/-} mice was utilized to differentiate innate immune cells. Different *in vitro* assays were performed with bone marrow-derived macrophages and myeloid dendritic cells and with isolated polymorphnuclear neutrophils, as well as T cells from respective mice. As mentioned earlier, bone marrow-derived macrophages (BMDMs) and bone marrow-derived myeloid dendritic cells (mDCs) are known to express Tec kinase family members Tec and Btk. Polymorphnuclear neutrophils (PMNs) express the family members Bmx, Tec and Btk while T cells do express Itk, Rlk and Tec.

3.2.1 Bone marrow analysis of Tec kinase-deficient mice

The majority of immune cells arise from precursor stem cells residing in the bone marrow of mammals (Abbas AK. et al, 2007). The composition of the bone marrow, therefore, constitutes an important “arsenal” to successfully fight infections. For example, *xid* mice show reduced numbers of cells of the myeloid lineage (e.g. Gr-1⁺ granulocytes and Gr-1⁻CD11b⁺ precursors) (Mangla A. et al, 2004).

We were therefore interested if differences in the bone-marrow composition exist between knock out mice and their wild-type littermates. In contrast to Mangla A. et al, we did not detect significant differences in levels of selected (pre) immune cells in Tec kinase-deficient bone marrow (Figure 3.9) when compared to wild-type controls. Hence, no changes in levels of Gr-1⁺ granulocytes or F4/80⁺ macrophages were detected (Figure 3.9 A). Based on CD45⁺ staining and respective cell sizes (Figure 3.9 C) no differences in amount/occurrence of major cell populations was observed (Figure 3.9 B). These data suggest that the difference in the immune response to *Candida* must come from stimulus-specific differentiation events triggered by fungal infections.

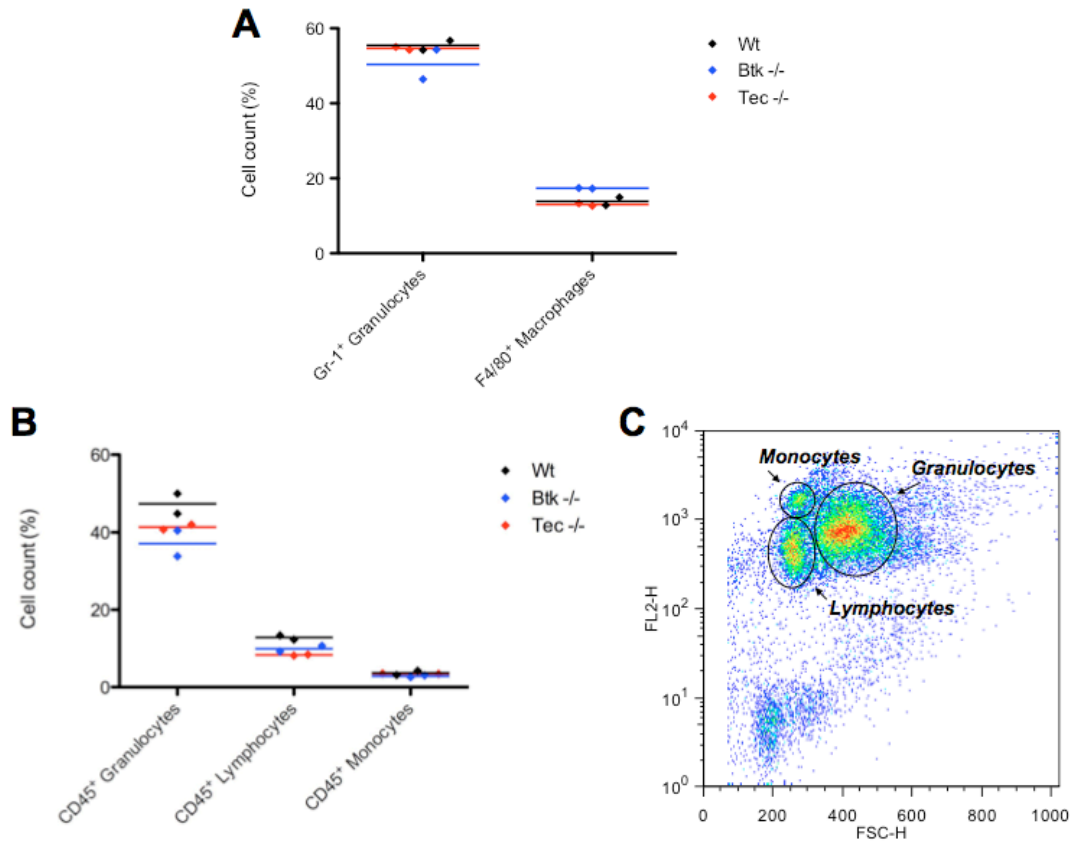


Figure 3.9: **Bone marrow composition of mice**

Femur and tibiae of mice were flushed and resulting cells were immediately stained for respective surface markers. FACS analysis was performed. Cell levels (%) are referred to 20 000 cells counted by FACS. (A) Gr-1⁺ granulocytes and F4/80⁺ macrophages; (B) CD45⁺ granulocytes, lymphocytes and monocytes; (C) FACS blot, showing different cell populations (sizes) of CD45⁺ cells.

3.2.2 *Tec family kinases and their role in BMDMs*

3.2.2.1 *Phagocytosis of Candida spp.*

Tec kinase family members have been reported to be involved in phagocytic processes (Jongstra-Bilen J. et al, 2008). We therefore hypothesised that phagocytosis of *Candida* spp. may be affected in *Tec*^{-/-} or *Btk*^{-/-} BMDMs. Notably, there was no significant difference in the phagocytosis of *Candida* spp. by Tec family kinase-deficient BMDMs when compared to WT BMDMs (Figure 3.10). Noteworthy, we could detect differences in the rate of phagocytosis between *Candida albicans* (around 40%) and *Candida glabrata* (around 70%).

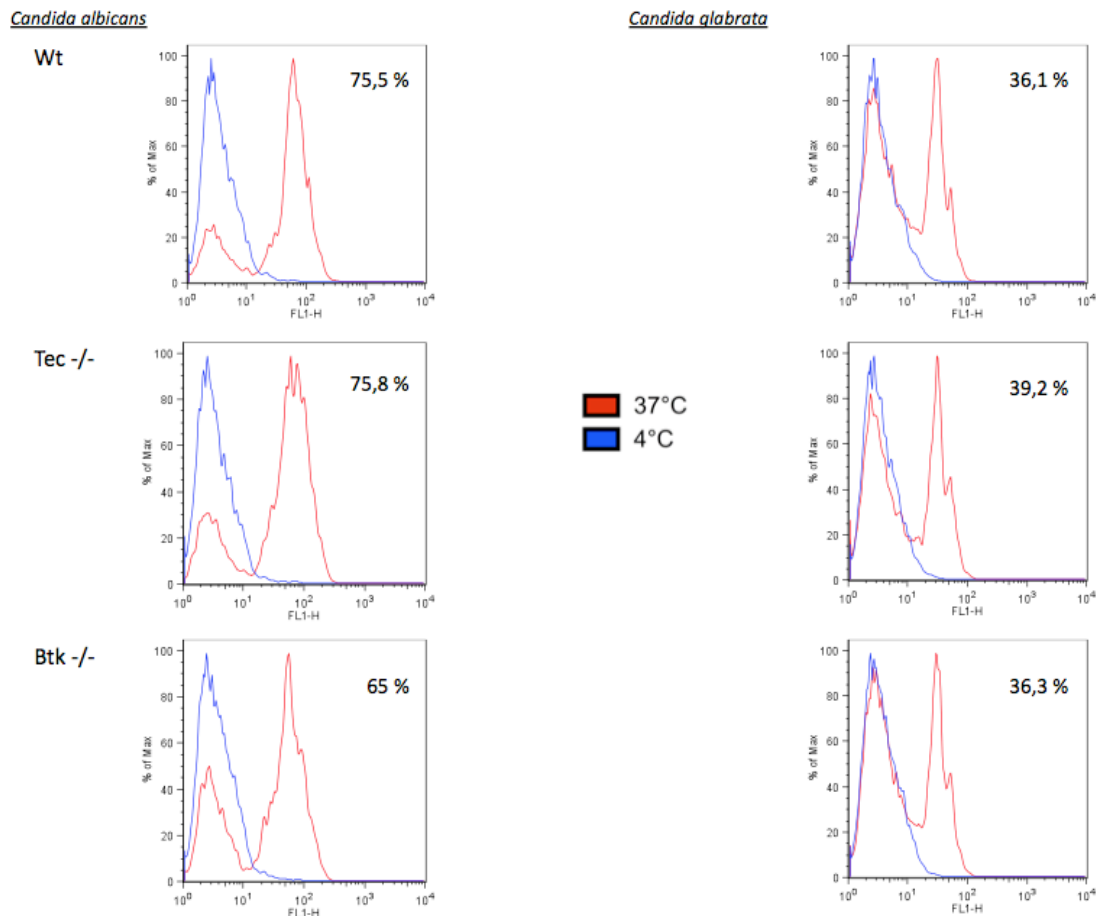


Figure 3.10: **Phagocytosis of *Candida* spp. in BMDMs**

BMDMs were incubated with FITC-labelled Ca or Cg for 45 Min at indicated temperatures. % of BMDMs having endocytosed at least 1 fungal cell were assessed by FACS analysis. Percentage of phagocytosis at 37°C are indicated. One representative of three independent experiments is shown.

3.2.2.2 Production of reactive oxygen species (ROS)

After phagocytosis of a pathogen, macrophages immediately start to activate microbicidal effector functions, including the production of reactive oxygen species (ROS) and nitric oxide intermediates (iNOS). Thus, this oxidative burst reaction plays an important role in the early killing of *Candida spp.* (Danley DL. et al, 1983; Frohner IE. et al, 2009). Defects in ROS production result in increased susceptibilities to bacterial and fungal infections (Aratani Y. et al, 2002).

We therefore wanted to know, if *Tec*^{-/-} and *Btk*^{-/-} BMDMs reveal any impairment in the induction of a ROS in response to *Candida spp.* Indeed, *Tec*^{-/-} and *Btk*^{-/-} BMDMs showed severe impairment of ROS production upon different fungal stimuli in comparison with WT BMDMs (Figure 3.2), measuring total ROS produced upon stimulation with *Candida spp.*. The highest reduction could be observed for macrophages stimulated with zymosan from *S. cerevisiae* (A). Interestingly, *Tec* family kinase-deficient macrophages show severe impairment of ROS when stimulated with *C. glabrata* (C), while cells stimulated with *C. albicans* (B) are still able to mount a – significantly – reduced response. No significant differences between *Tec*^{-/-} and *Btk*^{-/-} BMDMs in the induction of a ROS-response could be determined.

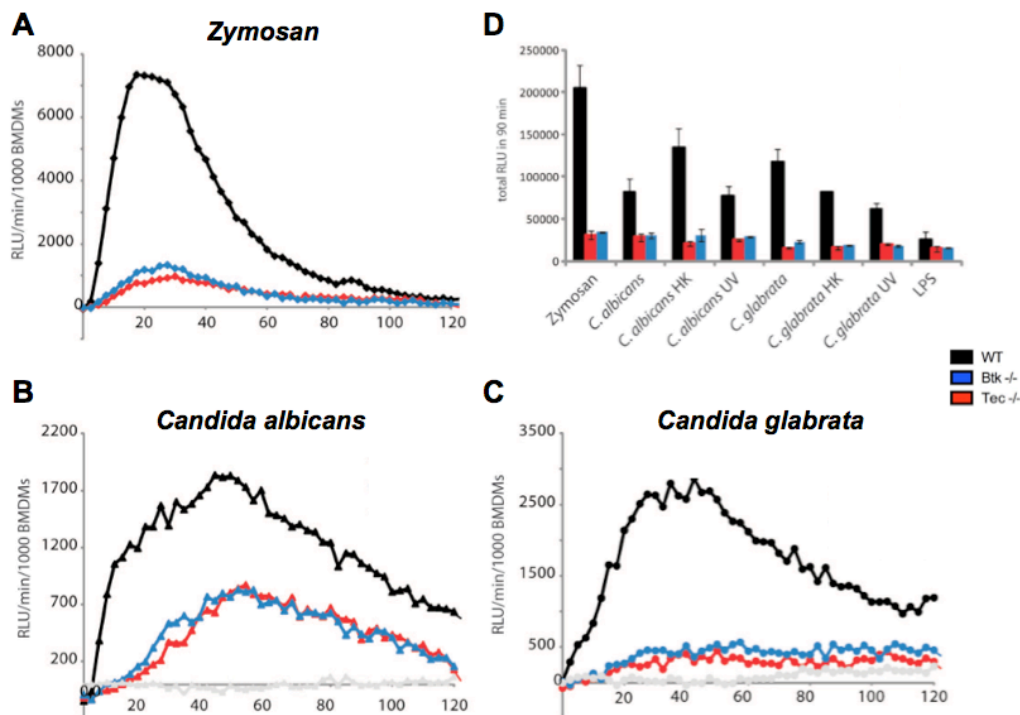


Figure 3.11: ROS production in response to fungal stimuli

BMDMs were loaded with 0,2mM Luminol and stimulated with indicated fungal stimuli (for *Candida*: MOI 5:1; Zymosan 100 µg/ml; LPS 1 µg/ml) for 120 Min. Increase in fluorescence was measured in real-time at 37°C. One representative of three independent experiments is shown.

3.2.2.3 *In vitro* killing of *C. albicans*

To assess the killing capacity of Tec family kinase-deficient BMDMs, an *in vitro* killing assay was performed. *C. albicans* was co-cultured with macrophages and after 2 days surviving *C. albicans* colonies were counted. Tec^{-/-} and Btk^{-/-} BMDMs showed reduced killing capacity of *C. albicans* when compared to WT BMDMs (Figure 3.12). Dynasore, a specific inhibitor of dynamin-mediated phagocytosis, completely inhibited the killing capacity of WT BMDMs, indicating that *C. albicans* killing by BMDMs requires phagocytosis. The decrease in killing capacity of Tec^{-/-} and Btk^{-/-} BMDMs is in agreement with the lower ROS-release observed in these cells in response to *C. albicans* infection (Figure 3.2.1.2).

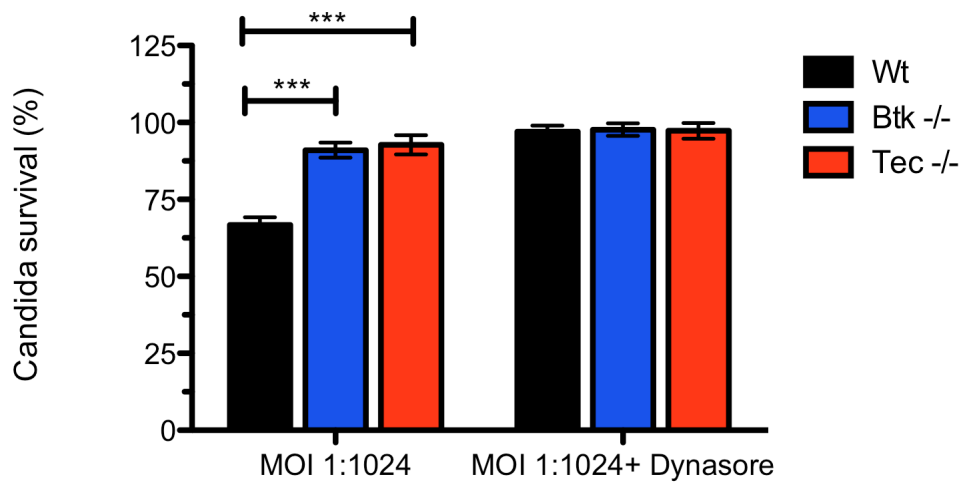


Figure 3.12: *In vitro* killing of *C. albicans* by BMDMs

BMDMs were co-cultured with *C. albicans* for two days at indicated MOIs with or without Dynasore (80 μ M). Afterwards, *Candida* colonies were stained and survival of *Candida* was assessed. Values of 3 independent experiments were pooled.

3.2.2.4 Induction of apoptosis

Tec family kinase-deficient BMDMs exhibit impaired M-CSF signalling and thus, show increased apoptosis (Melcher M. et al, 2008). Therefore, we hypothesised that Tec family kinase-deficient BMDMs stimulated with different fungal stimuli may display altered apoptosis responses. Additionally, high ROS production has also been associated with induction of apoptosis (Kasahara Y. et al, 1997). Hence, we speculated that reduced levels of ROS could also influence apoptosis of Tec family kinase-deficient BMDMs.

Interestingly, no significant differences in induction of apoptosis could be observed when compared to WT BMDMs (Figure 3.13). BMDMs were stimulated with puromycin (positive control), heat-killed *C. albicans* or zymosan. None of these stimuli revealed any changes in Tec^{-/-} or Btk^{-/-} BMDMs compared to WT BMDMs. After 8 hours, samples stimulated with puromycin exhibited a clear induction of apoptosis, measured by an increase in Caspase 3/7 levels. Cells stimulated with HKCa of zymosan failed to reveal any induction of apoptosis after 8 hours and 24 hours. However, the “Caspase 3/7 Glow Assay” is only able to measure apoptotic cells (cells that still have active caspase 3 and/or caspase 7) but it is unable to quantify the amount of already dead cells. Nevertheless, no time-dependent or genotype-dependent change in apoptosis of Tec^{-/-} or Btk^{-/-} BMDMs compared to WT cells could be observed.

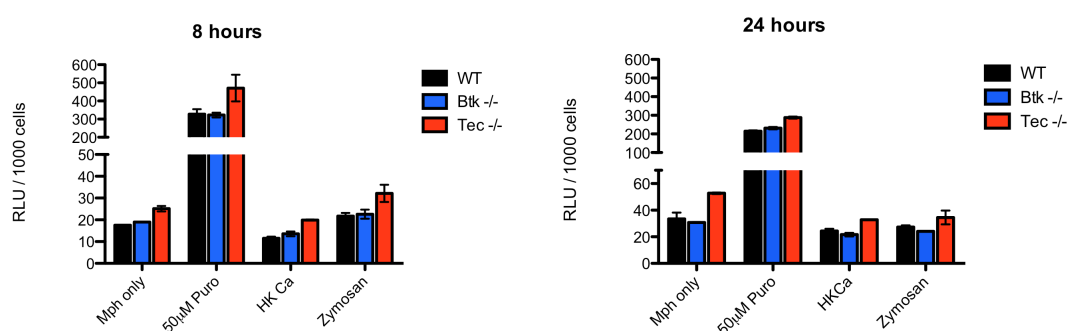


Figure 3.13: Induction of apoptosis in response to different fungal stimuli

BMDMs were stimulated with puromycin (50 µM), HKCa (MOI 5:1) or zymosan (100 µg/ml) for indicated time. Rate of apoptosis was measured by using Caspase 3/7 Glow Assay (Promega). Values of 2 independent experiments were pooled.

3.2.2.5 Cytokine and chemokine production in response to *Candida* spp.

Recognition of *Candida* PAMPs by macrophages activates several signaling-cascades and transcription programs, ultimately leading to cytokine and chemokine release (Bourgeois C. et al, 2010). We therefore wanted to know if loss of a Tec kinase family member affects the release of certain cytokine and chemokine patterns in response to stimulation with *Candida* spp.

Tec^{-/-} and Btk^{-/-} BMDMs showed severely impaired production of IL-12 and TNF α in response to different fungal stimuli (Figure 3.14). A 50% reduction in IL-12 release was observed for Tec family kinase-deficient BMDMs when compared to WT BMDMs in response to all the stimuli tested (Figure 3.14 A). Similarly, TNF α release was also decreased by about 50% in knock-out BMDMs compared to WT in response to nearly all stimuli, except for live *Candida glabrata* and UV-killed *C. albicans* (Figure 3.14 B). Indeed, Tec^{-/-} and Btk^{-/-} BMDMs release TNF α to a level comparable to WT when incubated with live *Candida glabrata*, No IFN γ , IL-6, IL-10, IL-4 or IL-23 (p19/p40) could be detected in supernatants of BMDMs stimulated with all the tested fungal stimuli.

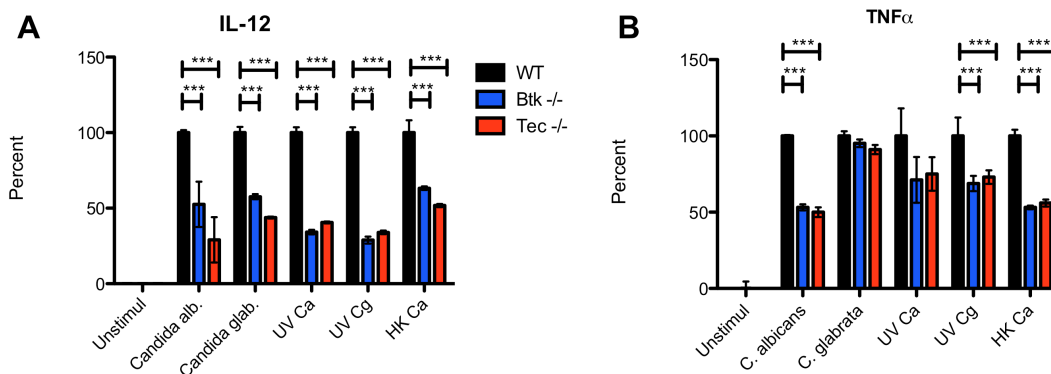


Figure 3.14: **Cytokines in response to fungal stimuli**

BMDMs were stimulated with either live or killed forms (UV, heat-killed) of *Candida* spp. (MOI 2:1) for 17 hours. Supernatants were taken and ELISAs were performed to quantify cytokines. Values are referred to respective WT-values. Values of 3 independent experiments were pooled.

All ELISA data were reconfirmed using a real-time PCR approach to measure RNA levels (Figure 3.15). Compared to wild-type controls, Tec^{-/-} and Btk^{-/-} BMDMs showed transcriptional downregulation of IL-12 and TNF α as also seen with ELISA. Additionally, IL-1 β , a classical pro-inflammatory cytokine, was also downregulated upon the loss of a Tec kinase family member.

IL-23 showed slight transcriptional downregulation. Interestingly, although IL-6 was undetectable in supernatants of cultures, it is transcriptionally upregulated in Tec^{-/-} and Btk^{-/-} BMDMs compared to WT BMDMs.

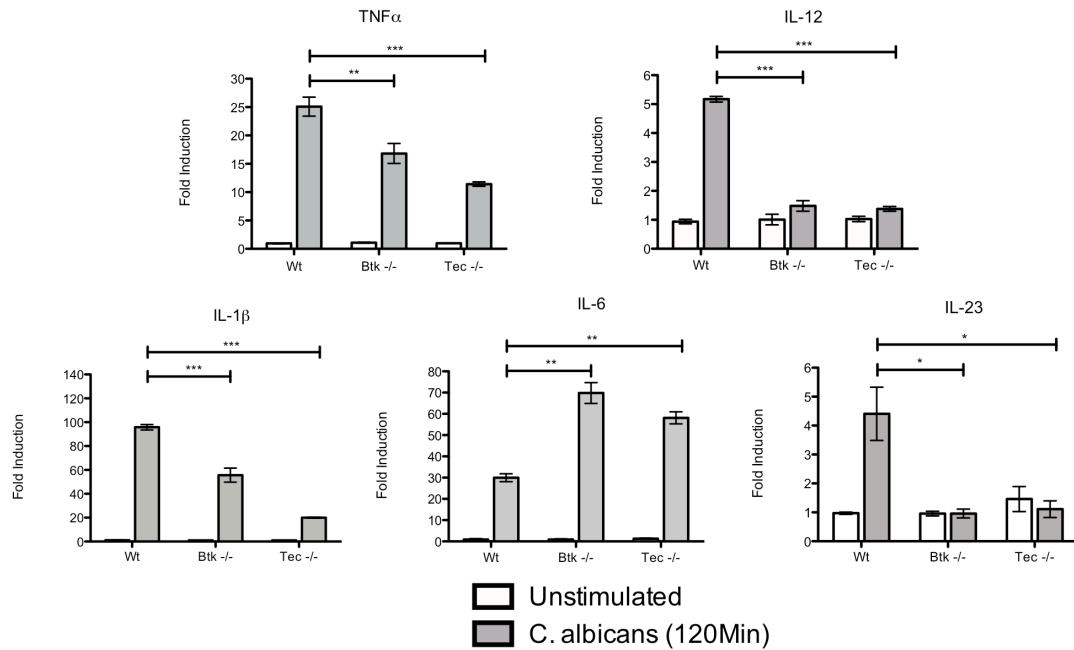


Figure 3.15: Cytokines in response to *C. albicans*

BMDMs were stimulated with *C. albicans* (MOI 2:1) for 120 Min. RNA samples were taken, reversely transcribed and real-time PCR was performed (house keeping gene: GAPDH; for primer sequences see Appendix). Values of 2 independent experiments were pooled.

Chemokines act as chemo-attractants, which are essential for the recruitment of different immune cells to site of infection (Abbas AK. et al, 2007). *In vitro* stimulation of BMDMs with *C. albicans* revealed a quite heterogeneous picture (Figure 3.16) qPCR indicated that chemokines like IP10, Rantes and Mip1a remained unaffected upon the loss of a Tec family kinase, MIP2 (attractant for neutrophils) and MIP1b (attractant for monocytes and natural killer cells) were transcriptionally upregulated. For MCP1 (attractant for monocytes and dendritic cells), Tec family kinase-deficient BMDMs exhibited transcriptional downregulation. Notably, KC (attractant for neutrophils) showed an exclusive transcriptional upregulation in Btk-deficient BMDMs.

Results

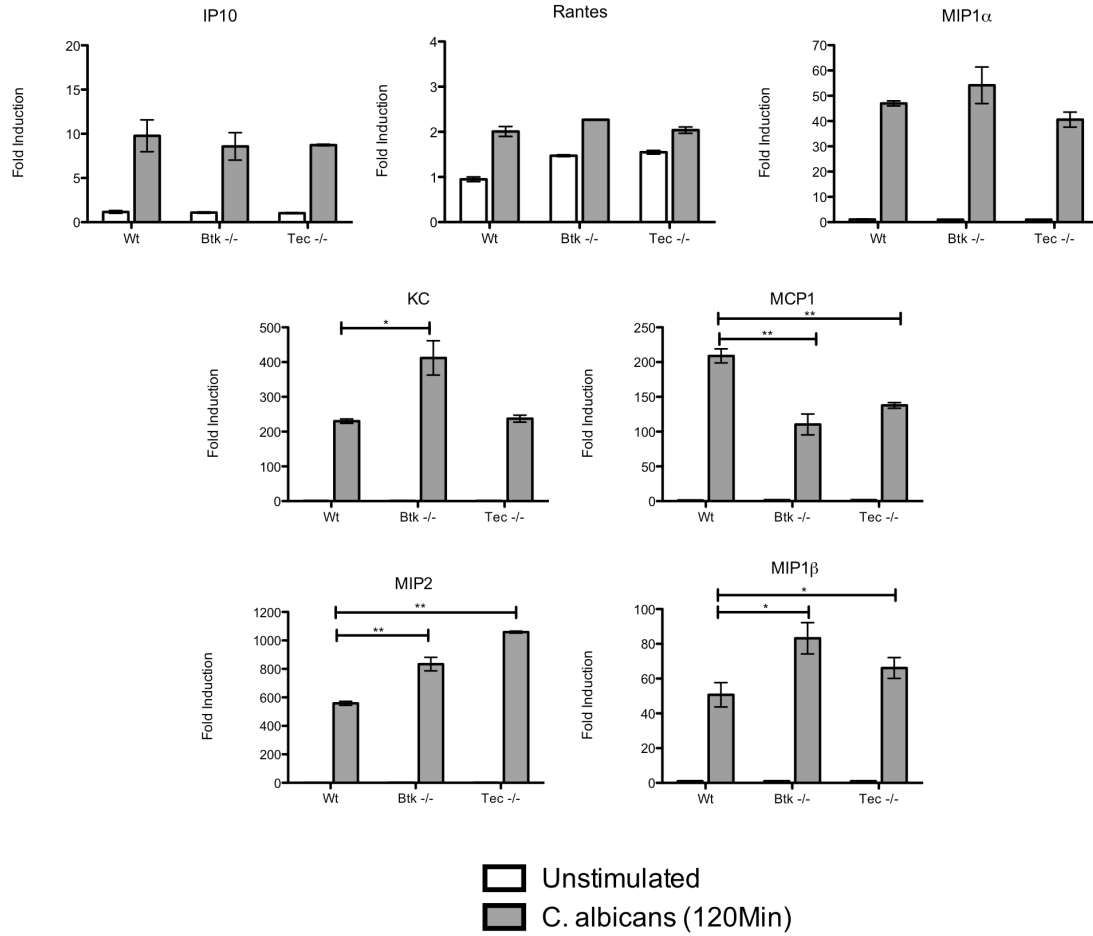


Figure 3.16: **Chemokines in response to *C. albicans***

BMDMs were stimulated with *C. albicans* (MOI 2:1) for 120 Min. RNA samples were taken, reversely transcribed and real-time PCR was performed (house keeping gene: GAPDH; for primer sequences see Appendix). Values of 2 independent experiments were pooled.

3.2.3 Tec Family Kinases and their role in mDCs

3.2.3.1 Phagocytosis of *Candida spp.*

Next, we were interested if Tec Kinase-deficient mDCs show any impairment in phagocytosis of *Candida spp.* Tec^{-/-} and Btk^{-/-} mDCs incubated with either *C. albicans* or *Candida glabrata* showed no significant differences in phagocytosis of respective fungus compared to WT mDCs (Figure 3.17). Notably, compared to BMDMs (Figure 3.10) mDCs showed higher rates of phagocytosis. Nonetheless, also in mDCs the phagocytosis of *C. albicans* was significantly lower than the phagocytosis of *C. glabrata*.

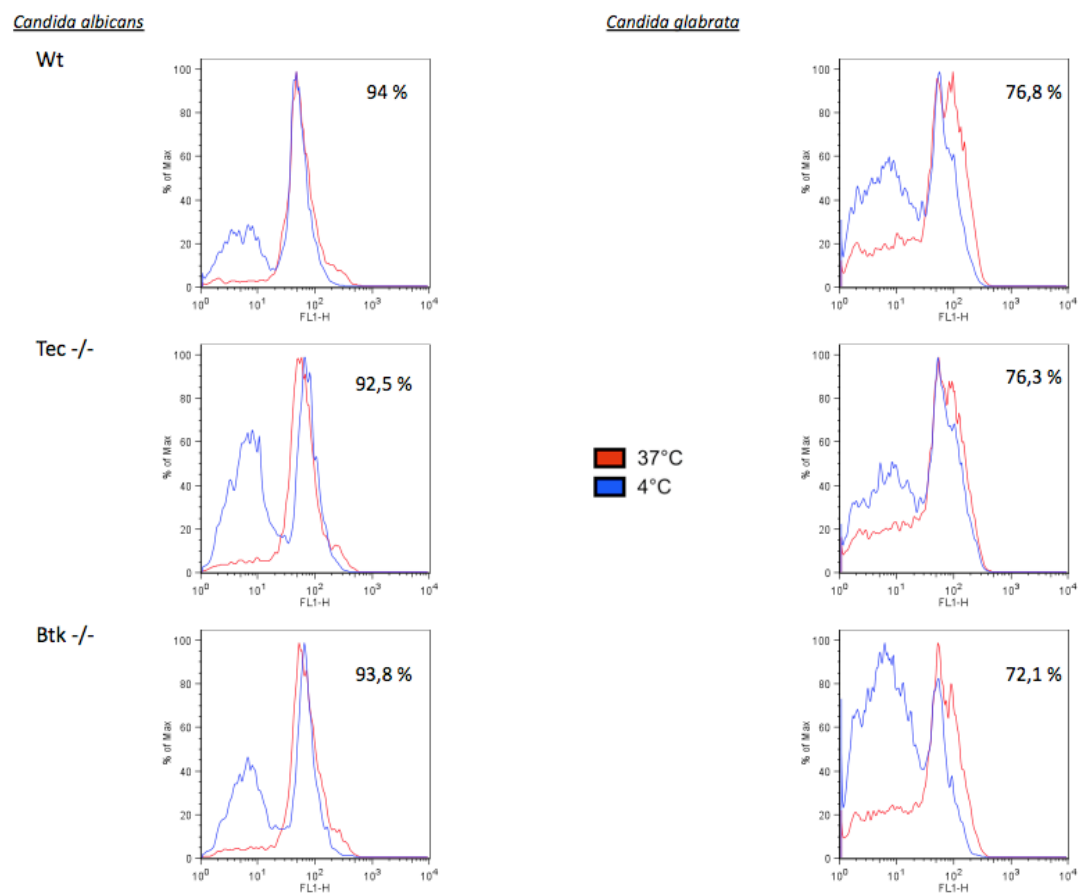


Figure 3.17: **Phagocytosis of *Candida spp.* in mDCs**

mDCs were incubated with FITC-labelled Ca or Cg for 45 Min at indicated temperatures. % of mDCs having endocytosed at least 1 fungal cell were assessed by FACS analysis. Percentage of phagocytosis at 37°C are indicated. One representative of three independent experiments is shown.

3.2.3.2 Activation of mDCs by *C. albicans*

After phagocytosis of a microbe, mDCs are immediately activated and start to fight the invading pathogen. During this process, mDCs upregulate different cell-surface markers including the costimulatory molecule CD80 (B7-1) (Peach RJ. et al, 1995), the costimulatory molecule CD86 (B7-2) (Chen C. et al, 1994), maturation marker CD83 (Zhou LJ. and Tedder TF, 1995) and the costimulatory molecule CD40 (Banchereau J. et al, 1994). Thus, the appearance of these cell-surface markers can be referred as activation status of mDCs.

We were therefore interested if Tec family kinases are involved in the activation of mDCs upon the stimulation with heat-killed *C. albicans*. For this purpose, we used a FACS-based assay. Stimulation of mDCs with heat-killed *C. albicans* resulted in a clear upregulation of previously described surface markers. Interestingly, no significant differences in surface expression of CD40, CD80, CD83 and CD86 was detected in Tec kinase-deficient mDCs compared to WT mDCs (Figure 3.18).

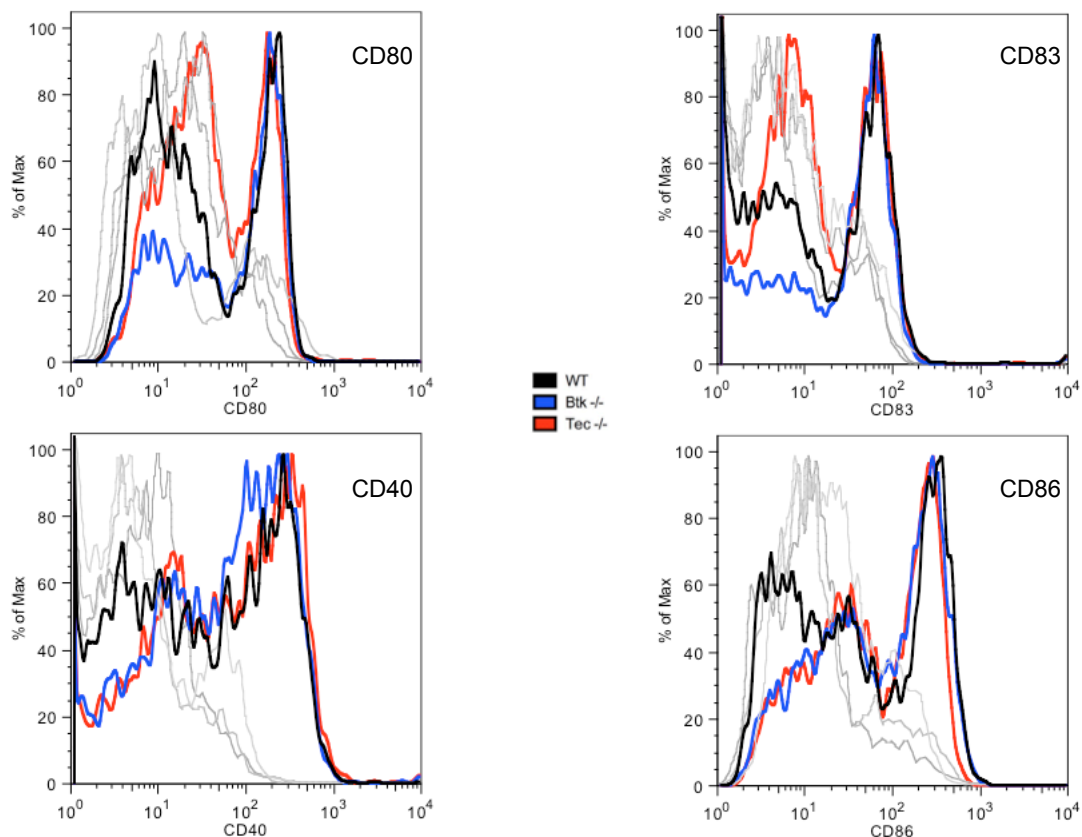


Figure 3.18: **Activation of mDCs in response to heat-killed *C. albicans***

mDCs were stimulated with HK-Ca for 17 hours (MOI 2:1). Cells were scraped off and stained with fluorescently labelled antibodies against CD40, CD80, CD83 or CD86. FACS analysis was performed. Grey lines indicate unstimulated mDCs.

3.2.3.3 **Cytokine production in response to *Candida* spp.**

After triggering various PRRs by *Candida* spp., dendritic cells start to produce and release a complex pattern of cytokines (Bourgeois C. et al, 2010). These signaling proteins are essential in initiating and coordinating immune responses. Additionally, cytokine production by dendritic cells is essential to provide naïve T cells a proper signal with instructions to differentiate into pathogen-specific, individual Th-lineages.

To address the question if the lack of a Tec kinase family member affects the cytokine environment provided by dendritic cells, mDCs of Tec^{-/-} respectively Btk^{-/-} mice were challenged with different fungal stimuli, and release of cytokines was quantified by ELISA (Figure 3.19). Upon challenge with live or inactivated *Candida*, no significant differences were seen concerning TNF α production of differently stimulated Tec^{-/-} and Btk^{-/-} mDCs. Stimulation of Tec^{-/-} and Btk^{-/-} cells with live and killed forms of *C. albicans* revealed only slightly decreased TNF α release (Figure 3.19 B).

However, Tec^{-/-} mDCs showed elevated levels of IL-6 production when stimulated with *C. albicans* and a tendency of elevated IL-6 release in response to heat-killed *Candida* spp. Interestingly, Btk^{-/-} mDCs exhibit no significant changes in IL-6 release upon stimulation with different fungal stimuli (Figure 3.19 A). For IL-10, Tec^{-/-} mDCs showed a clear elevated release upon stimulation with *Candida* spp., with striking differences for mDCs stimulated with heat-killed *C. albicans*. Noteworthy, Btk^{-/-} mDCs display elevated levels of IL-10 only when stimulated with *C. albicans* (live or heat-killed) (Figure 3.19 C).

For IL-23, the picture was not that clear: Tec^{-/-} mDCs responded by increased levels of IL-23. Again, striking differences for mDCs stimulated with live *C. albicans*, as also observed for Btk^{-/-} mDCs was seen (Figure 3.19 D).

In summary, cytokine response patterns in Tec kinase-deficient mDCs suggest changes in the cytokine environment provided by dendritic cells, most likely influencing T cell development (lineage decision).

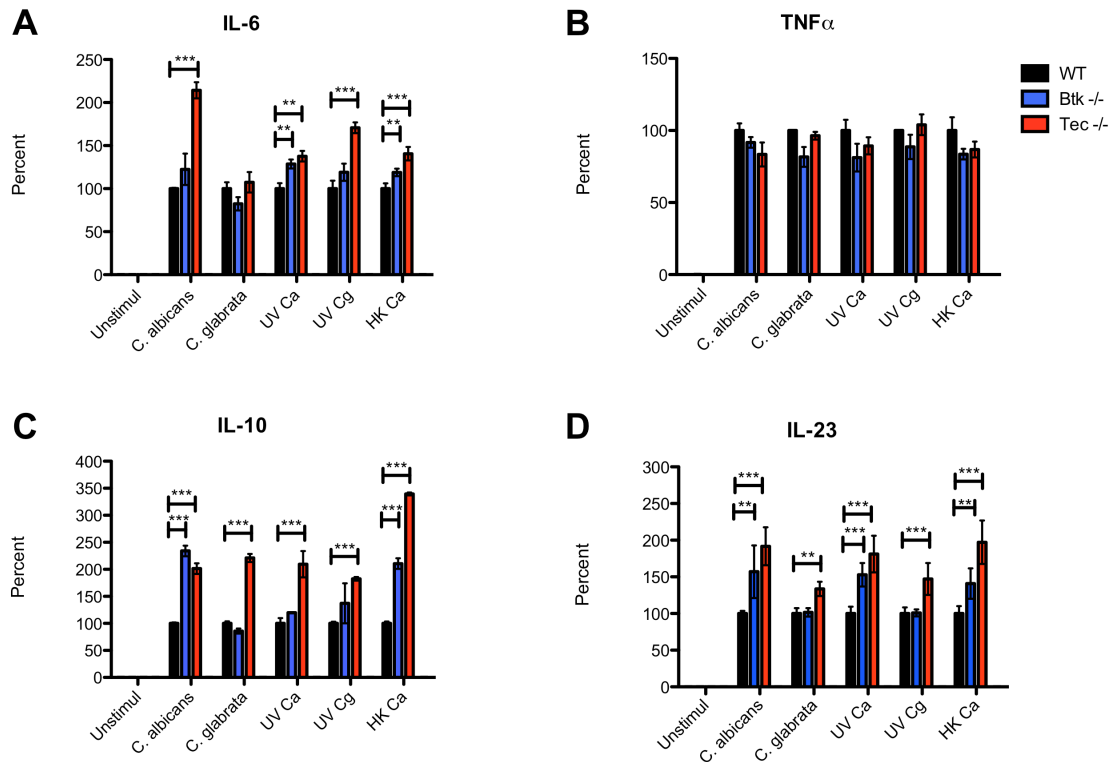


Figure 3.19: **Cytokine patterns in response to fungal stimuli**

mDCs were stimulated with either live or killed forms (UV, heat-killed) of *Candida spp.* (MOI 2:1) for 17 hours. Supernatants were taken and ELISAs were performed to quantify cytokines. Values are referred to respective WT-values. Values of 3 independent experiments were pooled.

3.2.3.4 MAP kinase signaling in response to *Candida spp.*

Next, we hypothesised that changes in cytokine pattern may likely arise from alterations in major intracellular signaling cascades. We therefore checked activation of the stress signaling pathways ERK 1/2, JNK and p38 with Western Blot.

Notably, stress signaling in Tec kinase-deficient mDCs was nearly unchanged in response to *Candida spp.* (Figure 3.20). Only JNK showed a slight increase in signal when compared to the wild-type controls. As previously shown (Majer Olivia, 2007), *C. albicans* induces a strong activation of ERK 1/2 and JNK, while *Candida glabrata* does not. However, p38 activation is induced equally independent of *Candida spp.*

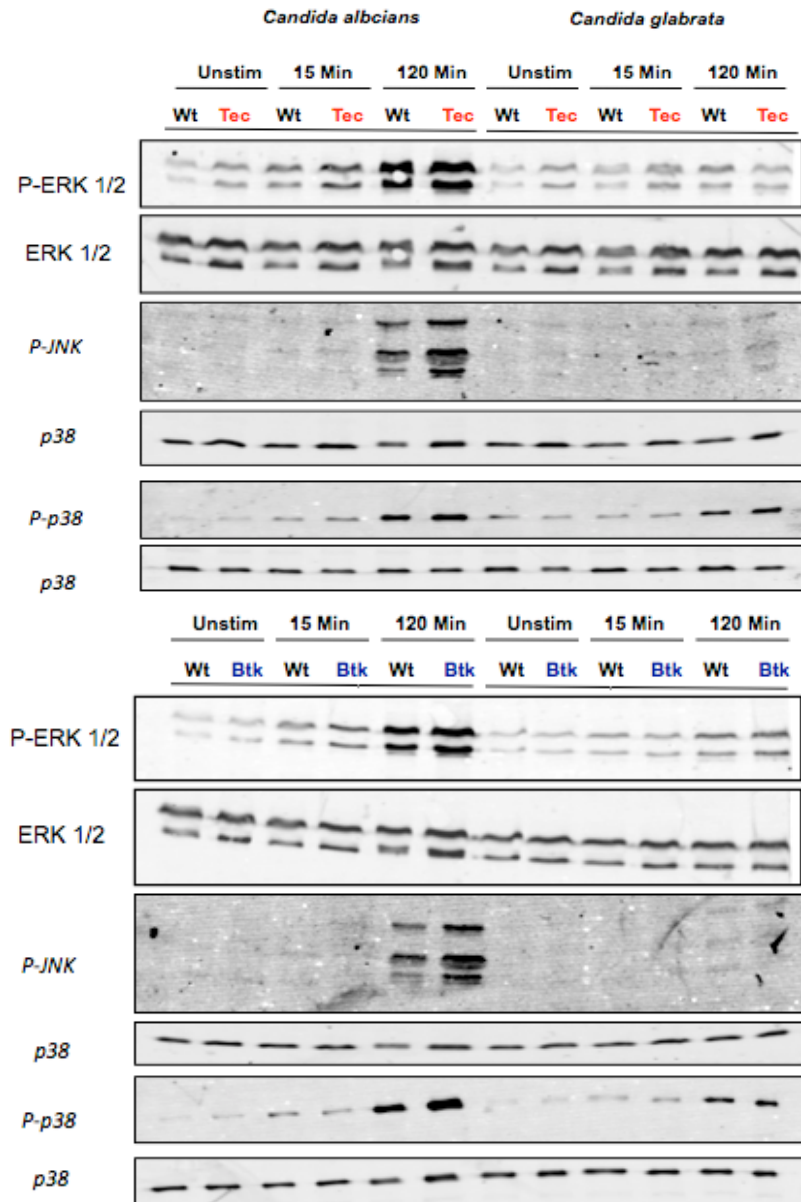


Figure 3.20: **Map kinase activation of mDCs in response to *Candida* spp.**

mDCs were stimulated with *Candida* spp. (MOI 2:1) for indicated times. Protein samples were taken and SDS-PAGE followed by immunoblot analysis was performed. Indicated antibodies were used (see Material and Methods) to detect activation of MAP kinase and to check for equal loading (loading control). One representative of two independent experiments is shown. Tec: Tec^{-/-}; Btk: Btk^{-/-}

3.2.3.5 STAT signaling in response to *Candida spp.*

Earlier studies suggested a reduced STAT3 activation of Btk-deficient mDCs in response to LPS, in an IL-10-dependent manner (Kawakami Y. et al, 2006). We therefore made the hypothesis that STAT activation may also be impaired in Tec kinase-deficient mDCs challenged with *Candida spp.* (Figure 3.21). The quantification of STAT activation in mDCs, stimulated with *C. albicans*, is quite complicated, since activation of STATs need at least 2 hours. During this time, the majority of mDCs are already damaged by hyphae of *C. albicans*. This is also the reason why nearly no STAT1 can be detected after 2 hours in the loading control lane.

For *Candida glabrata*, which exists only in the yeast form and does not damage mDCs in co-culture, no difference in activation of STAT1 was observed in Tec^{-/-} respectively Btk^{-/-} mDCs in comparison to WT controls. A tendency of higher activation of STAT3 in comparison with WT mDCs could be detected in Tec^{-/-} mDCs after 6 hours. This result is in line with higher IL-10 release in Tec^{-/-} mDCs stimulated with *Candida glabrata* in comparison with wild-type control (Figure 3.19).

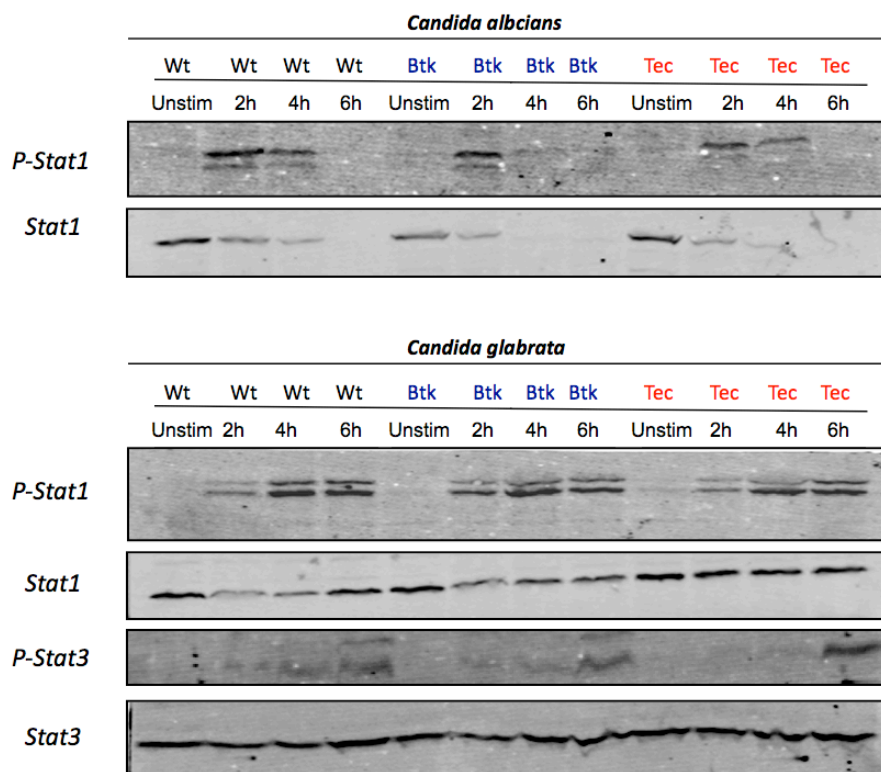


Figure 3.21: **Activation of STAT proteins in mDCs in response to *Candida spp.***

mDCs were stimulated with *Candida spp.* (MOI 2:1) for indicated time. Protein samples were taken and SDS-PAGE followed by Western Blot analysis was performed. Indicated antibodies were used (see Material and Methods) to detect activation of STAT proteins and to control equal loading (loading control). Tec: Tec^{-/-}; Btk: Btk^{-/-}

3.2.4 Tec Family Kinases and their Role in PMNs

3.2.4.1 *In vitro* killing of *C. albicans*

We further were interested if Tec^{-/-} as well as Btk^{-/-} PMNs show any impairment in killing of *C. albicans*. Interestingly, no significant differences in killing capacity between the Tec kinase-deficient and WT PMNs was detected (Figure 3.22). Noteworthy, PMNs show much higher killing capacity compared to macrophages (see Figure 3.12).

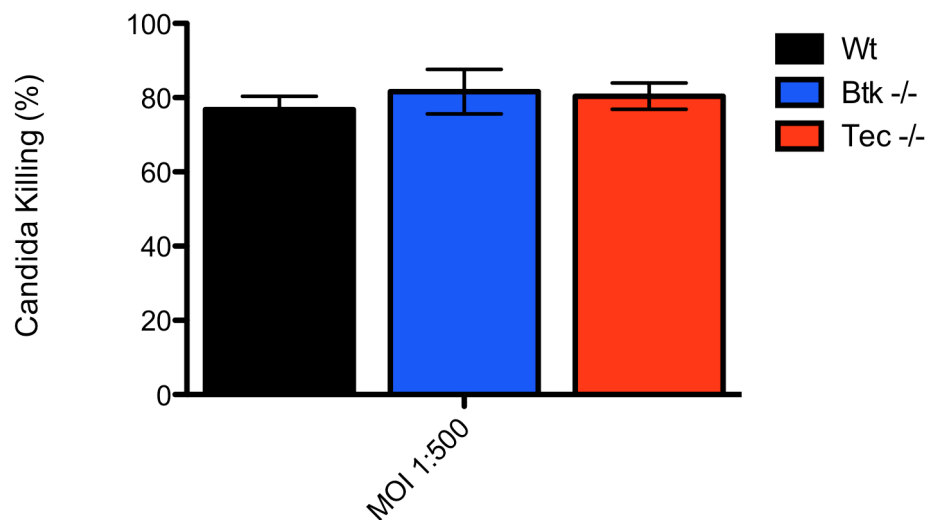


Figure 3.22: *In vitro* killing of *C. albicans* by PMNs

PMNs were co-cultured with *C. albicans* for two days (at different MOIs; ranging from 1:1-1:500). Afterwards, *Candida* colonies were stained and survival of *Candida* was assessed. Values of 2 independent experiments were pooled.

3.2.4.2 Cytokine production in response to fungal stimuli

Next, we wanted to test if *Tec*^{-/-}, respectively *Btk*^{-/-}, PMNs show impaired release of cytokines as have observed for *Tec* kinase-deficient BMDMs and mDCs. The release of different cytokines in the supernatants of challenged PMN-cultures was checked. Notably, the only detectable cytokine was *TNF*α. No *TNF*α could be detected in any of the PMNs-cultures stimulated with LPS or curdlan (a β-1,3-glucan). Additionally, *TNF*α release in response to live *C. albicans* is very low relative to BMDMs or mDCs. *Tec*^{-/-} respectively *Btk*^{-/-} PMNs showed significantly reduced production of *TNF*α upon challenge with heat-killed *C. albicans* or zymosan (e.g. *Btk*^{-/-}) (Figure 3.23). Other cytokines (IL-6, IL-10, e.g.) were undetectable in supernatants of stimulated cultures.

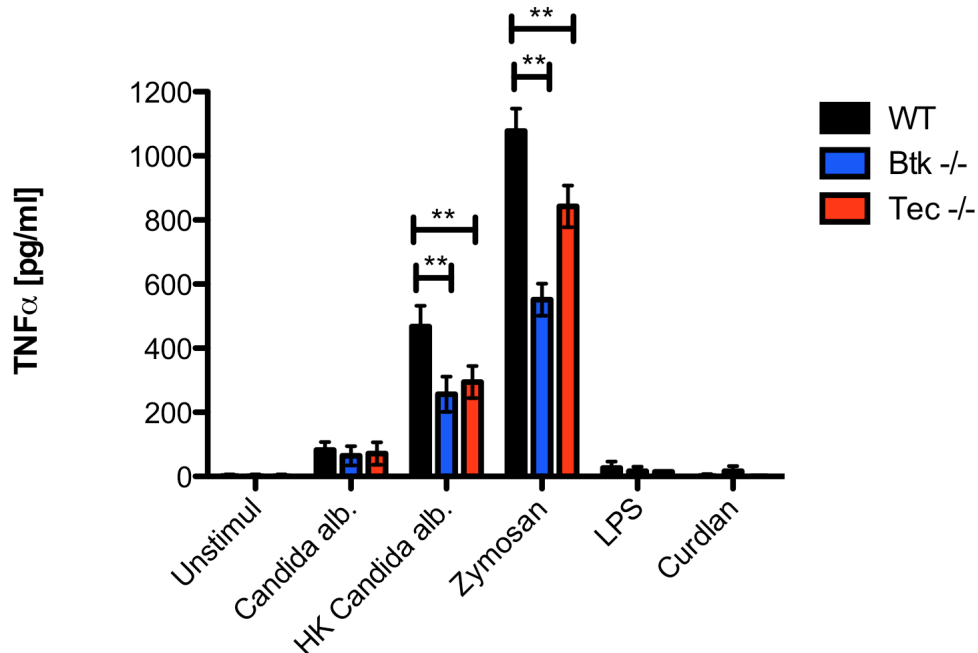


Figure 3.23: **Cytokine release in response to fungal stimuli**

PMNs were stimulated with either live or heat-killed *C. albicans* (MOI 2:1), LPS (1 μg/ml), zymosan (250 μg/ml) or curdlan (250 μg/ml) for 17 hours. Supernatants were taken and ELISAs were performed to measure cytokines. Values of 2 independent experiments were pooled.

3.2.5 Tec Family Kinases and their role in T cells

3.2.5.1 Restimulation of primed T cells

To address the question whether T cells from *Tec*^{-/-} mice show changes in differentiation or effector functions, we further analyzed the T cell response. Therefore, primed T cells were isolated from infected mice and restimulated *in vitro*. Spleens of infected mice (7 day p.i.; dose: 0,5 x 10⁵ CFU/mouse; i.v.), were collected and homogenized. From this tissue suspension, CD4⁺ T cells were isolated, resulting in >96% pure CD4⁺ T cell cultures. T cells were restimulated with plate-bound anti-CD3 ϵ and anti CD28 in the presence of GolgiPlug.

Since we were especially interested in CD3⁺CD4⁺CD25⁺ regulatory T cells, FACS analyses of restimulated primed T cells were performed (Figure 3.24). Notably, CD3⁺CD4⁺CD25⁺ T cells from *Tec*^{-/-} mice showed higher surface expression of CD25 after restimulation. Furthermore, restimulated WT T cells showed expression levels of non-restimulated *Tec*^{-/-} T cells, indicating a clear upregulation of CD25 in T cells of *Tec*^{-/-} mice.

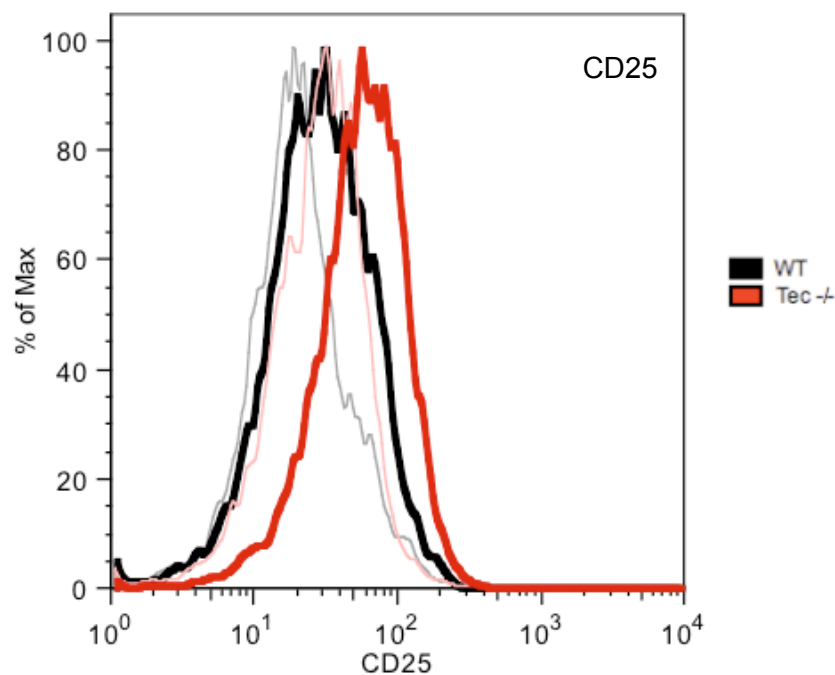


Figure 3.24: **CD25 expression of restimulated primed T cells**

WT and *Tec*^{-/-} mice (all C57BL/6) were challenged for 7 days with 0,5 x 10⁵ live *C. albicans* i.v. per mouse. After 7 days, spleens were taken, homogenized. CD4⁺ T cells were isolated and T cells from 5 mice/genotype were pooled. Cells were restimulated with 1 μ g/ml anti-CD3 ϵ and 3 μ g/ml anti-CD28 for 4 hours in the presence of GolgiPlug. T cells were stained for CD3, CD4 and CD25 and FACS analysis was performed on BD FACS Calibur. Thin lines in respective color indicate non-restimulated cells, thick lines indicate restimulated cells.

Restimulated primed T cells were also stained for intracellular Foxp3 expression, since Foxp3 is a hallmark of regulatory T cells (Hori S. et al, 2003). Thus, increased expression would indicate upregulation of a tolerogenic immune response in *Tec*^{-/-} mice (Figure 3.25).

Indeed, T cells of *Tec*^{-/-} mice showed slightly increased expression of Foxp3 in non-restimulated (Figure 3.25 A), as well as in restimulated (Figure 3.25 B) samples compared to levels of primed T cells from WT mice.

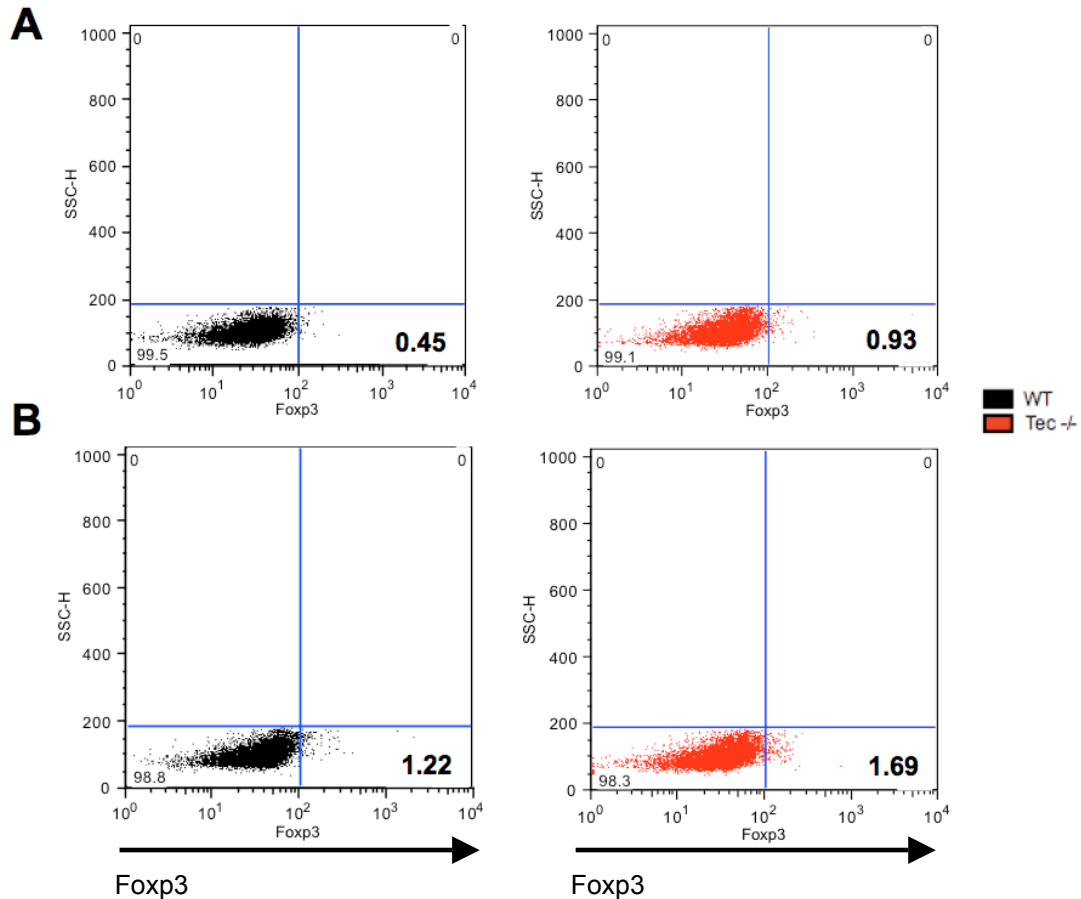


Figure 3.25: **Foxp3 expression of restimulated primed T cells**

WT and *Tec*^{-/-} mice (all C57BL/6) were challenged with 0.5×10^5 live *C. albicans* i.v. per mouse. After 7 days, spleens were taken, homogenized. CD4⁺ T cells were isolated and T cells from 5 mice/genotype were pooled. Cells were restimulated with 1 μ g/ml anti-CD3 ϵ and 3 μ g/ml anti-CD28 for 4 hours in the presence of GolgiPlug. T cells were stained for CD3, CD25 and Foxp3 and FACS analysis was performed on BD FACS Calibur. (A) non-restimulated T cells (B) restimulated T cells; Number in right lower corner represents % of CD3⁺CD25⁺Foxp3⁺ T cells

3.4.5 Dendritic cell / T cell co-culture

Since both dendritic cell and T cell data (3.2.3.3) indicated an enhanced tolerogenic immune response in the absence of Tec, we were interested in addressing the question which of the cell types – dendritic cells or T cells – from Tec^{-/-} mice contribute more/most to the observed regulatory response (esp. in crude splenocyte cultures). Hence, we established a co-culture system, which enables us to co-culture wild-type dendritic cells with Tec^{-/-} T cells (and vice versa). Therefore, we can specifically identify the cellular “source” of changed cytokine profiles.

Naïve and primed T cells from wild-type and Tec^{-/-} mice (7 day p.i.; dose: 0,5 x 10⁵ CFU/mouse; i.v.) were co-cultured with *in vitro* differentiated myeloid dendritic cells (wild-type respectively Tec^{-/-}). Cells were restimulated with UV-killed *C. albicans* and culture was incubated for 3 days. Supernatants were taken and signature-cytokines for each Th-lineage were measured (Figure 3.26).

Primed T cells of Tec^{-/-} mice seem to produce higher levels of IFN-γ when co-cultured with either wild-type or Tec^{-/-} mDCs when compared to the respective WT control. Additionally, WT T cells co-cultured with Tec^{-/-} mDCs also released elevated levels of IFN-γ (Figure 3.26 A). Comparing IFN-γ data from splenocyte restimulation experiments and these data, Tec^{-/-} samples exhibited elevated IFN-γ production relative to WT samples in both assays (Tec^{-/-} mDCs co-cultured with Tec^{-/-} T cells, respectively WT mDCs co-cultured with WT T cells). Increased IFN-γ production of Tec^{-/-} T cells was also observed in samples of mDCs co-cultured with naïve T cells, although total levels of IFN-γ were lower in cultures of naïve T cells (Figure 3.26 B). IFN-γ was exclusively produced by T cells, since mDCs alone stimulated with UV-killed *C. albicans* did not release any IFN-γ (Figure 3.26 C).

Strikingly, nearly the same pattern is also true for IL-17. Primed T cells of Tec^{-/-} mice produced higher levels of IL-17 when co-cultured with either wild-type or Tec^{-/-} mDCs. Wild-type T cells co-cultured with Tec^{-/-} mDCs also released elevated levels of IL-17 (Figure 3.26 A). Co-culture experiments revealed a much more pronounced Th17 response in Tec^{-/-} mice than observed in splenocyte restimulation assays. Significantly increased IL-17 production of Tec^{-/-} T cells was also observed in samples of mDCs co-cultured with naïve T cells, although total levels of IL-17 were lower (Figure 3.26 B). IL-17 was exclusively produced by T cells, since mDCs alone stimulated with UV-killed *C. albicans* failed to release any IL-17 (Figure 3.26 C).

Levels of released IL-10 revealed no relevant differences between WT and Tec^{-/-} samples (Figure 3.26 A+B). Nevertheless there was a tendency of increased IL-10 in samples with primed and naïve Tec^{-/-} T cells. Interestingly, the highly significant elevated IL-10 level in splenocyte restimulation experiments in Tec^{-/-} was not be detected in the co-culture assay.

Since mDCs also produce IL-10 (Figure 3.26 C) in response to UV-killed *C. albicans*, measured IL-10 in co-culture samples cannot reflect IL-10 release by T cells only. Notably, wild-type and Tec^{-/-} mDCs released nearly equal levels of IL-10 in response to *C. albicans*, which is in contrast to results reported in 3.2.3.3 and Figure 3.19. However, results of these experiments cannot be compared, since mDCs in co-culture experiments were stimulated for 72 hours, therefore, these values do and can not reflect values of samples after 17 hours of stimulation (stability of IL-10 in supernatant, kinetics of IL-10 production e.g.).

Finally, IL-2 has been reported as a hallmark of T cell responses, since proliferating T cells release high amounts of IL-2 (Abbas AK. et al, 2007). Notably, the development of regulatory T cells is highly dependent on IL-2. However, it is currently unclear whether IL-2 is also important for their suppressive function *in vivo* (Barron L. et al, 2010). Furthermore, the tolerogenic effect of regulatory T cells is to dampen T cell responses, inhibit proliferation of T cells and, therefore, decrease release of IL-2 (Abbas AK. et al, 2007). Strikingly, primed Tec^{-/-} T cells co-cultured with Tec^{-/-} mDCs exhibit strongly decreased IL-2 production compared to wild-type samples, suggesting a dampened T cell response.

In conclusion, the DC / T cell co-culture suggests an increase in IL-17 production and therefore also in TH17 cells in the absence of Tec. This effect is dependent on mDCs as well as on T cells, indicating a mDC- and T cell-intrinsic phenotype upon the loss of the Tec kinase family member Tec.

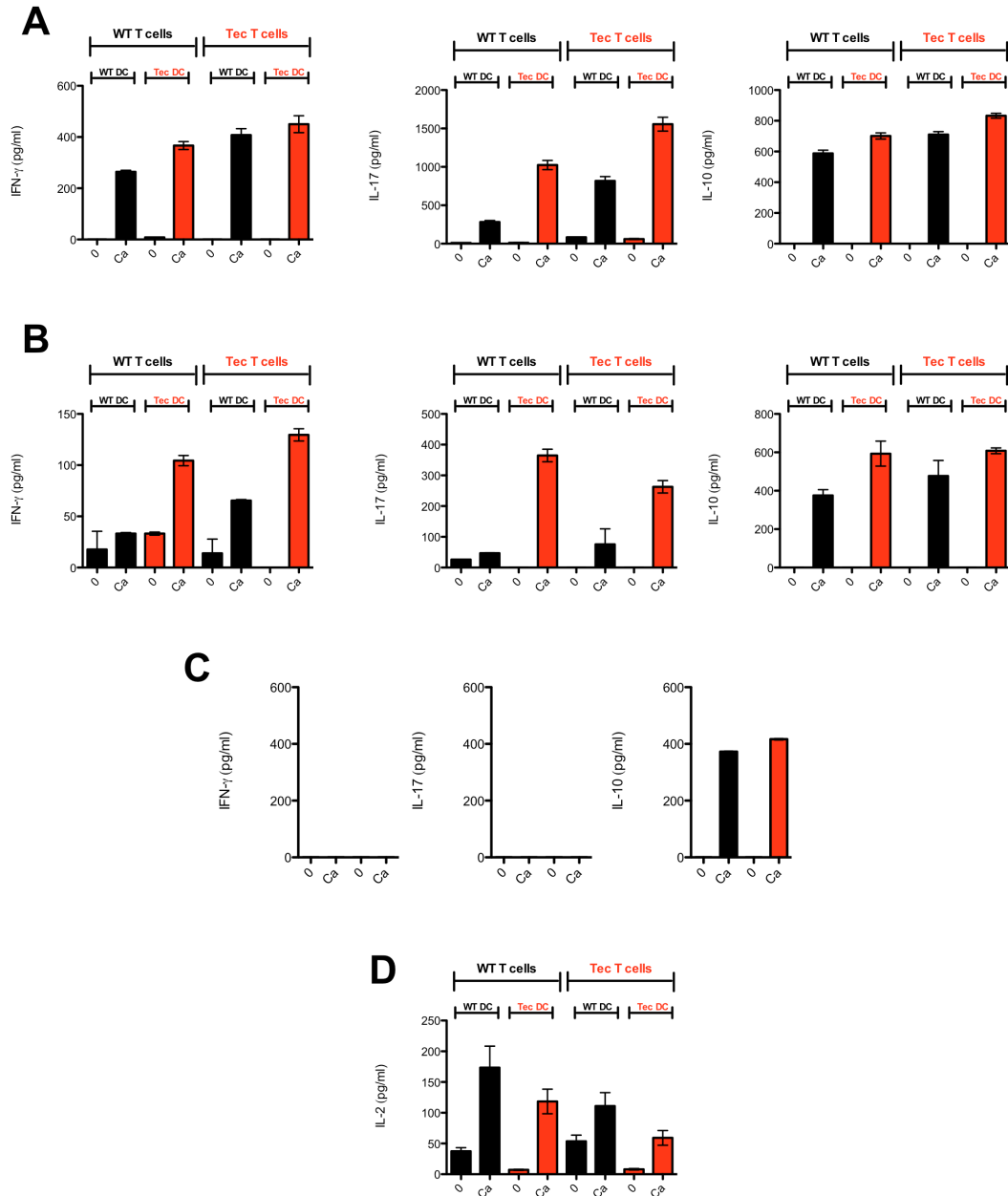


Figure 3.26: **Dendritic cell / T cell co-culture**

WT and Tec^{-/-} mice (all C57BL/6) were challenged with 0,5 x 10⁵ live *C. albicans* i.v. per mouse. After 7 days, spleens were taken, homogenized. CD4⁺ T cells were isolated and T cells from 5 mice/genotype were pooled. Naïve T cells were obtained from PBS-control mice. Differentiated DCs were infected with UV-killed *C. albicans* (MOI 1:1) and T cells were added to the DCs (MOI 5:1). Co-culture was incubated 72 hours. Supernatants were taken and released cytokines were measured with ELISA. (A) Primed T cells; (B) Naïve T cells; (C) DCs only (D) IL-2 release of primed T cells. Released IL-4 was under the detection limit in all different cultures.

4 DISCUSSION

In this study, we report an improved survival of Tec *-/-* mice to a challenge by fungal pathogens such as *Candida albicans*. Thus, we demonstrate for the first time a role of Tec family kinases in fungal pathogenesis. Using *in vitro* models of immune cells / *Candida* interactions, we elucidate some of the molecular mechanisms of Tec kinase function.

Improved outcome for the host is not due to improved pathogen clearance

Increased fungal burdens in Tec *-/-* and Btk *-/-* mice compared to their wild-type littermates are likely to arise from impaired functions of immune cells, which are apparently unable to kill the invaded fungal cells. Especially innate immune cells are normally known as the main killing effector cells (Abbas AK. et al, 2007). Therefore, fungal burdens are consistent with results observed for BMDMs: Tec kinase-deficient BMDMs show significant decreased killing capacity of *Candida albicans in vitro* (Figure 3.12). These results also clearly demonstrate that impaired *in vitro* killing of *Candida albicans* by Tec kinase-deficient BMDMs is not due to reduced phagocytic properties of respective BMDMs (Figure 3.10). Although Tec family kinases have been reported to participate in phagocytosis (Jongstra-Bilen J. et al, 2008), the uptake of fungal cells (*Candida albicans* as well as *Candida glabrata*) is not affected by the loss of one Tec kinase family member. Either the other remaining active Tec kinase member (Tec resp. Btk) is able to compensate for the loss of the missing kinase or Tec family kinases are not involved in the *Candida*-phagocytosis at all. Since reactive oxygen species (ROS) are important for the clearance of *Candida albicans* (Frohner IE. et al, 2009), we investigated the ROS response in Tec kinase-deficient BMDMs. Indeed, Tec *-/-* and Btk *-/-* BMDMs exhibited impaired production of ROS when stimulated with a variety of different fungal stimuli (Figure 3.11). Our results are in line with a report showing reduced levels of reactive oxygen intermediates (ROIs) in *xid* macrophages stimulated with PMA. Interestingly, there seems to exist differences in the involvement of Tec family kinases in the induction of ROS, depending on the fungal species. While Tec kinase-deficient BMDMs stimulated with *C. albicans* are still able to mount a reduced ROS response, knock-out cells stimulated with *C. glabrata* exhibit a severe impairment in the production of ROS. This might implicate different mechanisms in the induction of ROS (i.e. different surface receptors) for different *Candida spp.*, and therefore distinct roles of Tec family kinases. Hence, the impaired killing of *Candida albicans* in Tec kinase-

deficient BMDMs (Figure 3.12) may likely arise from altered production of ROS by BMDMs in these mice.

Noteworthy, Tec kinase-deficient PMNs do not exhibit any defects in killing of *C. albicans in vitro* (Figure 3.22), indicating that neutrophils cannot explain increased colony-forming units in infected mice. The remaining two Tec kinase family members in neutrophils (Btk and Bmx and Tec and Bmx, respectively) may fully compensate for the loss of a single family member. Alternatively, our results could also indicate that Tec kinases are not involved at all in pathways that activate killing mechanisms of *Candida albicans* in neutrophils – this question has to be addressed in future experiments.

High production of ROS has been associated with elevated induction of apoptosis (Kasahara Y. et al, 1997). However, although Tec^{-/-} and Btk^{-/-} BMDMs produced less ROS, their survival to *Candida* infection was not increased when compared to Wt cells (Figure 3.12). Notably, *xid* BMDMs reveal impaired inflammation-induced apoptosis upon stimulation with LPS or IFN- γ in an nitric oxide- and ROS-independent manner (Khare A. et al, 2011). Interestingly, a reported LPS-induced apoptosis can not be observed for samples stimulated with *Candida*, indicating, that inflammation-induced apoptosis is either a *xid*-specific feature absent in Btk^{-/-} BMDMs or stimulation with LPS triggers much more inflammatory pathways than *Candida* does. Consequently, cells are more likely to enter apoptosis.

In conclusion, increased fungal burdens observed in Tec kinase-deficient mice may likely arise from an impaired oxidative burst, and thus impaired killing capacity of Tec^{-/-} and Btk^{-/-} macrophages. Tec kinase-deficient BMDMs reveal impaired ROS-production, but no changes in phagocytic properties and induction of apoptosis upon infection with *Candida spp*. These results therefore suggest that Tec kinases trigger signaling downstream of receptors that lead to the production of ROS.

Decreased pro-inflammatory response

Systemic infections of mice with *C. albicans* lead to massive infiltrations of immune cell into the kidneys (Lionakis MS. et al, 2011). However, these infiltrates also promote detrimental sepsis by releasing high amounts of pro-inflammatory cytokines (Spellberg B. et al, 2005). Our study provide evidence of a decreased pro-inflammatory response in *Candida*-infected *Tec*^{-/-} mice and respective immune cells, which are entirely consistent with their improved survival to systemic fungal sepsis.

Candida-stimulated *Tec*^{-/-} and *Btk*^{-/-} BMDMs exhibit impaired production of pro-inflammatory cytokines (TNF α , IL-12, IL-1 β). A decreased release of TNF α from knock-out BMDMs is observed for LPS-stimulated *xid* BMDMs (Mukhopadhyay S. et al, 2002). Interestingly, TNF α production of BMDMs stimulated with live *C. glabrata* exhibited no differences between knock-out and Wt cells, implying a Cg-specific, Tec kinase-independent pathway of TNF α production. The good correlation between protein-concentration and transcript induction indicates that impaired release of inflammatory cytokines of *Tec*^{-/-} and *Btk*^{-/-} BMDMs results from decreased transcription of those cytokines compared to wild-type cells. Thus, Tec kinases seem to be involved in pathways that mediate or promote release of inflammatory cytokines in response to fungal stimulation. Hence, a loss of a Tec kinase family member leads to reduced transcriptional activation of cytokine target genes.

These cytokine results are consistent with a report, demonstrating the involvement of Btk in MHCII-dependent TLR signaling. Indeed, Btk-deficient peritoneal macrophages show impaired release of inflammatory cytokines upon stimulation with various TLR ligands in comparison to Wt peritoneal macrophages (Liu X. et al, 2011).

In addition, *Tec*^{-/-} and *Btk*^{-/-} neutrophils show significantly decreased levels of TNF α production in response to heat-killed *C. albicans* and zymosan when compared to wild-type controls (Figure 3.23). These results implicate Tec family kinases in the induction of TNF α release, consistent with results observed for BMDMs. Further, data indicate that Tec and Btk show redundant functions in TNF α production in response to heat-killed *C. albicans* and zymosan, since release of TNF α in *Tec*^{-/-} and *Btk*^{-/-} PMNs occurs at similar levels.

Interestingly, levels of TNF α seem similar between Tec kinase-deficient and wild-type mDCs, which is in contrast to results observed for BMDMs and PMNs. These data therefore indicate a Tec kinase-independent pathway of TNF α -release in response to *Candida spp.* in myeloid dendritic cells. It may be the first evidence for a

cell specificity of Tec family kinases in different innate immune cells. We also show here that the activation levels of major MAP-Kinase cascades are unchanged upon challenge with *Candida spp.* in Tec^{-/-} and Btk^{-/-} mDCs when compared to their wild-type controls. Thus Tec kinases are probably not involved in major MAP-Kinase signalling cascades. Tec kinase family members participate in MAP-Kinase-independent signaling pathways, such as those induced by several C-type lectin-like receptors (Bourgeois C. et al, 2010).

Our results show a clear decrease of pro-inflammatory cytokines in Tec^{-/-} and Btk^{-/-} BMDMs and PMNs upon *Candida* challenge, suggesting that innate immune cells with the exception of mDCs lacking these kinases have reduced pro-inflammatory properties. However, it has to be mentioned that the systemic levels of pro-inflammatory cytokine IL6 is unchanged between wild-type and Tec kinase-deficient mice after 7 days of infection with *C. albicans*, suggesting that the renal cytokine response is more critical for the disease outcome than the systemic immune answer.

The chemokine response mounted by innate immune cells is essential for the recruitment of additional immune cells, and therefore for the orchestration of an immune answer. Hence, changes in this pattern will influence the cellular composition at the site of infection, and ultimately also affect the survival of the host. Interestingly, the chemokine-pattern of Tec^{-/-} and Btk^{-/-} BMDMs in response to *C. albicans* is changed when compared to wild-type controls. Tec^{-/-} and Btk^{-/-} cells exhibit increased expression of MIP2 (CXCL2; chemotactic for PMNs and hematopoietic stem cells; Abbas AK. et al, 2007) and MIP1b (CCL4; chemotactic for NK cells and monocytes; Abbas AK. et al, 2007) and a decreased expression of MCP1 (CCL2; chemotactic for inflammatory monocytes; Henderson AB. et al, 2003). Noteworthy, recent work from Majer and colleagues shows that reduced infiltration of inflammatory monocytes into the kidneys of *Candida*-infected mice correlates with better infection-outcome for the host (Majer O. et al, 2011). In addition, Btk^{-/-} cells display significant increase in KC-expression (CXCL1; chemotactic for PMNs) compared to Tec^{-/-} and Wt BMDMs.

Interestingly, we identify IP-10, Rantes and MIP1a as chemokines, which showed Tec kinase-independent expression in response to *Candida albicans*. The relevance of these chemokines has to be further studied.

In conclusion, *in vitro* *C. albicans*-stimulated Tec^{-/-} and Btk^{-/-} BMDMs exhibit an altered chemokine response profile, indicating a distinct immune cell recruitment *in vivo* in knock-out mice compared to wild-type mice. The reduced immune cell infiltrates into kidneys could explain the improved survival of Tec^{-/-}.

An altered adaptive immune response

Dendritic cells (DCs) are essential in generating a cytokine environment upon activation, which is necessary and crucial for the development and differentiation of the distinct T helper cell lineages. Thus, changes in the cytokine environment provided by DCs will influence the nature and magnitude of the T cell response (Abbas AK. et al, 2007).

Hence, striking differences between wild-type and Tec^{-/-} and Btk^{-/-} mDCs are observed in the cytokine environment produced upon stimulation with live and inactivated *Candida spp.* Tec^{-/-} mDCs release much higher levels of IL-6, IL-10 and IL-23 compared to wild-type cells, especially when stimulated with live *C. albicans*. In general, Tec^{-/-} mDCs seem to produce increased amounts of IL-10 compared to wild-type controls when stimulated with live and inactivated *Candida spp.*, implicating that Tec modulates or down-regulates signaling cascades that lead to IL-10 release in mDCs. Additionally, elevated activation of STAT3, a downstream signaling component of the IL-10 receptor, is observed in Tec^{-/-} mDCs. However, phosphorylation of ERK, which is activated to induce IL-10 production (Saraiva M. and O'Garra A., 2010), reveals no differences between *Candida*-stimulated Tec kinase-deficient and wild-type mDCs. Thus, the effect of Tec cannot be at the levels of MAP-kinases.

Btk^{-/-} mDCs, however, do not exhibit such drastic changes in their pattern of released cytokines when compared to Wt and especially to Tec^{-/-} mDCs. Hence, Tec is much more involved in signaling cascades activated by *Candida spp.* than Btk.

Thus, the cytokine environment provided by Tec^{-/-} mDCs in response to live *C. albicans* is likely to favor the development of Th17 and Tregs since it has been shown that IL-6 and IL-23 are potent inducers of Th17 cells and IL-10 of Tregs (Jetten AM., 2009).

To confirm this hypothesis and to further investigate the adaptive immune response in Tec kinase-deficient and Wt mice, splenocytes cultures were used. Restimulated splenocytes from Tec^{-/-} mice show exclusive upregulation of IL-10 and IFN- γ release compared to splenocytes from Wt mice, indicating a polarization towards the

respective Th-lineages (IL-10 for Treg and IFN- γ for Th1). Restimulated Tec^{-/-} splenocytes also exhibited a tendency to increased IL-17 production, although not significant. Thus, data obtained from restimulated splenocytes from Tec^{-/-} mice are consistent with the signature cytokines of Tec^{-/-} mDC cultures.

The highly upregulated IL-10 production in Tec^{-/-} mDCs and splenocytes in response to Ca are the first hints to further investigate a possible tolerogenic immune response induced by systemic infections with *C. albicans*. Romani and Puccetti reported about a IL-10-dependent mechanism, which allows *C. albicans* to “highjack” the immune system and enables the fungus to persist in distinct pathogenic niches within the host (Romani L. and Puccetti P., 2006). The key-molecule for this mechanism is indolamine 2,3-dioxygenase (IDO). However, neither restimulated Wt nor Tec^{-/-} or Btk^{-/-} splenocytes show any upregulation of IDO, indicating that increased IL-10 levels in Tec^{-/-} splenocytes arise from an IDO-independent mechanism.

Recently, Pulandran and colleagues discovered the molecular mechanism of a Vitamin A-dependent, tolerogenic pathway in dendritic cells, which strongly induces the proliferation and differentiation of Tregs and the production of IL-10 (Pulendran B. et al, 2010). Interestingly, Tec^{-/-} splenocytes exhibit the exclusive upregulation of each of the involved signalling components when compared to Wt and Btk^{-/-} splenocytes, indicating that this tolerogenic pathway may operate in spleens of Ca-challenged Tec^{-/-} mice.

The suppressor of cytokine signaling (SOCS) proteins have also been associated with dampening of immune responses. Remarkably, pathogens hijack these signaling proteins to evade immune surveillance (Dimitriou ID. et al, 2008). Interestingly, restimulated splenocytes of Tec^{-/-} mice display exclusive transcriptional upregulation of SOCS1, SOCS2, SOCS3 and SOCS4 when compared to Wt controls and Btk-deficient splenocytes. This is strong support of our hypothesis of an increased induction of a tolerogenic immune response in Tec^{-/-} mice upon challenge with *Candida albicans*.

The characterization of splenocyte cultures from Wt versus Tec^{-/-} mice lead us to the conclusion that the spleen-composition of Tec^{-/-} mice is quite comparable to Wt mice, with no major changes in the cellular composition in absence of pathogens. However, upon infection significant differences are observed for B cells, implying that a higher number of B cells migrate into spleens of Tec^{-/-} mice upon infection with *C. albicans*. Since B cells are prominent producers of IL-10 (Gieni RS et al, 1997), elevated IL-10 levels in restimulated Tec^{-/-} splenocyte cultures may also arise from B cells. However, the most striking differences are observed for regulatory T cells. Tec^{-/-} splenocytes display significant higher levels of CD25⁺ Tregs, which goes in line with observed IL-10 production of restimulated splenocytes and with RT-PCR data of induced tolerogenic immune programs. Additionally, these data receive further support from restimulation experiments of primed T cells. T cells of Tec-deficient mice show higher amounts of CD25 surface expression and Foxp3-induction upon restimulation compared to T cells from Wt mice. In conclusion, we demonstrate that Tec^{-/-} mice display a remarkable upregulation of IL-10-producing effector cells of the T- and B-cell lineage in response to infection with *C. albicans* suggesting an involvement of Tec in the recruitment, differentiation and activation of these cell types during infections caused by fungal pathogens.

DC/T cell co-culture experiments showed a significant increase in IL-17 production from primed and naive Tec^{-/-} T cells compared to wild-type cells. This effect is probably not only T cell-intrinsic since the co-cultivation of Tec^{-/-} T cells and Tec^{-/-} mDCs even boosts the production of IL-17. Thus, Tec^{-/-} mDCs might favor the development of Th17 cells, as already evidenced by mDCs *in vitro* cytokine data, Tec-deficient T cells display an intrinsic impairment in IL-17 production. Noteworthy, these data are consistent with a recent report showing that Tec-deficient mice display a special Th17-subset (CD44^{high}CD62L⁻), which produces enhanced levels of IL-17 upon restimulation with heat-killed *Streptococcus pneumoniae* (Boucheron N. et al, 2010). Hence, further experiments should address the question whether this particular Th17-subset is the major producer of IL-17 in *in vitro* and *in vivo* experiments with *C. albicans*. Restimulation experiments indicate an upregulation of Th17- and Treg-cells in *Candida*-challenged Tec^{-/-} mice. Our results together with other publications further support the hypothesis that the development and differentiation of Th17- and Treg-lineages are tightly linked, balanced and reciprocally regulated (Lee YK. et al, 2009; Kimura A. and Kishimoto T, 2010; Weaver CT. and Hatton RD, 2009). Interestingly, a link exists between the loss of a well-defined Th17/regulatory T cell balance during Simian Immunodeficiency Virus (SIV) infection, leading to impaired

survival of infected monkeys and indicating the importance of this cellular balance *in vivo* (Favre, D. et al, 2009).

Furthermore, a very recent publication reports about the dependency of Th17 and regulatory T cells. Regulatory T cells are able to induce the differentiation of Th17 cells and IL-17 production *in vivo* and *in vitro*. This mechanism is IL-2-consuming and enhances resistance of mice in oral infections with *C. albicans* (Pandiyani P. et al, 2011). Strikingly, we show that upregulation of Th17 and regulatory T cells, decreased levels of “free” IL-2 from Tec-deficient DC/T cell co-cultures as well as improved survival of systemically infected Tec^{-/-}. Further experiments should therefore in particular address the induction of this mechanism in Tec^{-/-} mice, also trying also to answer the question whether upregulation of regulatory T cells is triggered as a negative feedback in response to elevated Th17 cells, so as to dampen the immune response. Alternatively, regulatory T cells themselves could be the source of increased IL-17 in Tec-deficient animals.

Interestingly, Tec-deficient survivor-mice still show high fungal burdens in their kidneys, indicating that these mice encounter an improved survival even with fungal persistence. Notably, regulatory T cells are the major actors in the induction of tolerance and fungal persistence (De Luca A. et al, 2007). Since T cell data clearly indicate the upregulation of regulatory T cells in Tec^{-/-} mice, this mechanism is likely to be induced in Tec-deficient mice. Additionally, BUN-levels of infected Tec^{-/-} mice are significantly lower than in wild-type animals, further arguing for a tolerogenic immune response, which reduces tissue inflammation. However, it has to be mentioned that Tec^{-/-} survivor mice still exhibited a highly damaged kidney, possibly indicating that the tolerogenic immune response cannot prevent tissue damage but the induction of sepsis.

Tec versus Btk

Noteworthy, one of the most surprising findings of this thesis was the fact that Tec kinase family members Tec and Btk seem to have immune cell-specific, non-redundant functions, which may also be reflected in altered immune responses in *Candida*-infected mice. Systemically infected Btk-deficient mice reveal lower activation of an adaptive immune response, as indicated by significantly lower weights of the spleen of respective mice when compared to Tec^{-/-} and Wt mice. However, Btk-deficient mice have significantly elevated fungal burdens as Tec^{-/-} mice. Additionally, restimulated splenocytes of Btk^{-/-} mice only display slightly increased release of IFN- γ . Our results further indicate that none of the tested tolerogenic immune responses was induced in spleens of Btk^{-/-} mice, which is in

contrast to results observed for the Tec-deficient mice. Hence, the differences in the adaptive immune response between Tec^{-/-} and Btk^{-/-} mice may be alleageable by the altered cytokine responses mounted in DCs (esp. IL-6) in response to *C. albicans*. This environment is likely to enable Tec-deficient mice to polarize towards an increased Th17- and regulatory T cell response (Th17/Treg balance!).

Further *in vivo* and *in vitro* experiments should therefore address the question what mechanistic differences lead to the altered adaptive immune responses in Tec^{-/-} and Btk^{-/-} mice, and whether these mechanistic changes lead to impaired survival to *C. albicans* infections of Btk-deficient mice compared to Wt mice.

This work provides for the first time clear evidence that Tec family kinases are involved in the innate and adaptive immune response during infections with *Candida spp.* Effector functions of Tec kinase-deficient innate immune cells are strongly impaired. In particular, Tec^{-/-} dendritic cells exhibit a drastically changed cytokine environment in response to *Candida spp.* This study reports for the first time that Tec kinase family member Tec exerts cell-specific and non-redundant functions in myeloid dendritic cells challenged with fungal pathogens. Furthermore this thesis provides evidences, that Tec family kinases play a critical role in the host response to fungal infections by using an mouse model of systemic *C. albicans* infection. Tec kinase family member Tec seems to be involved in mechanisms shaping both Th17 and regulatory T cell responses. One may now speculate that in Tec^{-/-} mice the increased levels of Th17 cells are able to restrain the *Candida* infection, while the simultaneously induced regulatory T cells are able to dampen tissue inflammation, yet also prevent a full fungal clearance. This change in the Th17/Treg balance may explain the improved survival of Tec^{-/-} mice, which seem not to develop sepsis in response to infection with *Candida albicans*.

Further *in vivo* experiments are underway to address the question whether Tec^{-/-} mice are indeed sepsis-resistant, which cell type(s) are responsible for this phenotype and which role the Th17/Treg balance may play in this process.

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6 APPENDIX

Antibodies

Antibody	Dilution	Company
rabbit α -ERK (p42/p44)	1 : 1000	Cell Signaling Technology
rabbit α -p38	1 : 1000	Santa Cruz
rabbit α -JNK	1 : 1000	Cell Signaling Technology
rabbit α -phospho ERK (p42/p44)	1 : 1000	Cell Signaling Technology
rabbit α -phospho p38	1 : 1000	Cell Signaling Technology
rabbit α -phospho JNK	1 : 1000	Cell Signaling Technology
rabbit α - STAT1 (C term)	1 : 1000	Gift of Pavel Kovarik
rabbit α - STAT3	1 : 1000	Cell Signaling Technology
rabbit α -phospho STAT1	1 : 1000	Cell Signaling Technology
rabbit α -phospho STAT3	1 : 1000	Cell Signaling Technology

Primers

Primer	Sequence
mGAPDH_fwd	CATGGCCTTCCGTGTTTCCTA
mGAPDH_rev	GCGGCACGTCAGATCCA
mTNFa_fwd	CAAAATTCGAGTGACAAGCCTG
mTNFa_rev	GAGATCCATGCCGTTGGC
mIL1b_fwd	CAACCAACAAGTGATATTCTCCATG
mIL1b_rev	GATCCACACTCTCCAGCTGCA
mIL6f_fwd	GAGGATACCACTCCCAACAGACC
mIL6_rev	AAGTGCATCATCGTTGTTTCATACA
mIL23p19_fwd	GCAGATTCCAAGCCTCAGTC
mIL23p19_rev	TTCAACATATGCAGGTCCCA
mIL12p40_fwd	GGAAGCACGGCAGCAGAATA
mIL12p40_rev	AACTTGAGGGAGAAGTAGGAATGG
mSOCS3_fwd	AACTTGCTGTGGGTGACCAT
mSOCS3_rev	AAGGCCGGAGATTTTCGCT
mCcl2_fwd	ATTGGGATCATCTTGCTGGT
mCcl2_rev	CCTGCTGTTACAGTTGCC
mCcl5_fwd	CCACTTCTTCTCTGGGTTGG
mCcl5_rev	GTGCCCACGTCAAGGAGTAT
mCxcl2_fwd	TCCAGGTCAGTTAGCCTTGC
mCxcl2_rev	CGGTCAAAAAGTTTGCCTTG
mCxcl10_fwd	CCTATGGCCCTCATTCTCAC
mCxcl10_rev	CTCATCCTGCTGGGTCTGAG
mCxcl1_fwd	TCTCCGTTACTTGGGGACAC
mCxcl1_rev	CCACACTCAAGAATGGTCGC
mIDO_fwd	GTTCTCAATCAGCACAGGCA
mIDO_rev	GATGTGGGCTTTGCTCTACC
mSOCS4_fwd	GAGGAAGTTTCTTCTGGGCAC
mSOCS4_rev	GTGATGGCGGTGGTGATG
mSOCS6_fwd	CAGTTATGCCCAAACATCGG
mSOCS6_rev	TGCCTCCATAAGATTTGCCT
mCIS(H)_fwd	ACAGCCAGCAAAGGACAAG
mCIS(H)_rev	CTCCTTTCTCCTTCCATCCC
mSOCS1_fwd	ACAAGCTGCTACAACCAGGG
mSOCS1_rev	ACTTCTGGCTGGAGACCTCA
mSOCS2_fwd	AAGAAAGTTCCTTCTGGAGCC
mSOCS2_rev	CGCGAGCTCAGTCAAACAG
mRALDH2_fwd	AATCCCTAAATGGCGGTA
mRALDH2_rev	ATGGGCTCGTGTCTTGTG
mTGFB_fwd	TGACGTCACTGGAGTTGTACGG
mTGFB_rev	GGTTCATGTCATGGATGGTGC
Foxp3_fwd	CCTCTAGCAGTCCACTTCAC
Foxp3_rev	CTTCTTCTGTCTGGAGTGG

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University studies

October 2005 – present “Molecular Biology” at the University of Vienna

December 2008 First Academic Stage (1.Diplomprüfung), degree with first class honors

Practical trainings and Research Experience

April 2007 – 3 years part time job at the Institute of Medical Biochemistry, Max F. Perutz Laboratories, Vienna, Austria, Prof. Dr. Karl Kuchler

August 2008 – 2 month internship at the Institute of Molecular Medicine, Max F. Perutz Laboratories, Vienna, Austria, Prof. Dr. Karl Kuchler. *“Cloning of Nucleotide Binding Domains (NBD) of different ATP-Binding Cassette (ABC) Transporters in Saccharomyces cerevisiae”*

August 2009 – 1 month internship at the Institute of Biochemistry II, University of Cologne - Faculty of Medicine, Cologne, Germany, Prof. Dr. Manuel Koch. *“Role of Collagen XXII in Formation of Focal Contacts at the Myotendinous Junction”*

October 2009 – 1 month internship at the Department of Molecular Cell Biology, Max F. Perutz Laboratories, Vienna, Austria, Prof. Dr. Gerhard Wiche. *“Co-Immunoprecipitation and Immunofluorescence Analyses revealing the Interaction of Plectin with the eukaryotic Transcriptionfactor EF1gamma”*

February 2010 – 2 month internship at the Institute of Molecular Medicine, Max F. Perutz Laboratories, Vienna, Austria, Prof. Dr. Karl Kuchler. *“Establishment of RT-qPCR for Analysis of different Th Cell Lineages during Infection with Candida albicans”*

January 2011 – 12 month (ongoing) diploma thesis at the Institute of Medical Biochemistry, Christian Doppler Laboratory for Infection Biology, Max F. Perutz Laboratories, Vienna, Austria, Prof. Dr. Karl Kuchler. *“Function of Tec Family Kinases in the Innate Immune Response to Fungal Pathogens”*

3 semesters tutorial assistant at University of Vienna, Vienna, Austria – Practical Course *“Molecular Biology IIB”* and *“Laboratory Course in Biochemistry”*

Courses

November 2010, “Medical Mycology” organized by the Austrian Society for Medical Mycology, AKH Vienna

September & November 2010 “FELASA category B education” and “Animal reproduction course” (in vitro fertilization, embryo implantation) organized by the Veterinary University of Vienna

Oral & Poster Presentations

3rd - 5th December 2010, Poster presentation, ÖGAI Meeting “Function of Tec Family Kinases in the Innate Immune Response to Fungal Pathogens”, Vienna

7th-13th May 2011, Poster presentation, 4th FEBS Advanced Lecture Course Human Fungal Pathogens 2011: “The non-receptor tyrosine kinase Tec1 is implicated in regulating macrophage immune effector function in response to fungal pathogens”, La Colle sur Loup

8rd – 10th December 2011, Oral presentation, Meeting of Austrian Society of Medical Mycology “Function of Tec Family Kinases in the Innate Immune Response to Fungal Pathogens”, Vienna

Honors & Awards

2010, FEBS Youth Travel Fund for the Fourth **FEBS Advanced Lecture Course on Human Fungal Pathogens 2011**

Experience in Congress Organization

2007, part of the Organizer Team at the **32nd FEBS Congress: Molecular Machines** with up to 600 participants in Vienna/Austria in July 2007

2008, part of the Organizer Team at the **2nd FEBS Special Meeting on ABC Proteins** (ABC Proteins: From Multidrug Resistance to Genetic Disease) with up to 350 participants in Innsbruck/Austria in March 2008

2010, part of the Organizer Team at the **3rd FEBS Special Meeting on ABC Proteins** (ABC Proteins: From Multidrug Resistance to Genetic Disease) with up to 350 participants in Innsbruck/Austria in March 2010