Fakultät für Medizin
Abteilung für Hämatologie und internistische Onkologie

Charakterisierung der Rolle von XIAP in der Modulation des entzündlichen Zelltodes und dessen Konsequenzen

Nicole Cornelia Müller

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigten Dissertation

Vorsitzende(r):	Prof. Dr. Jürgen Ruland
Prüfer der Dissertation:	1. apl. Prof. Dr. Philipp Jost
	2. Prof. Dr. Dr. h.c. Horst Kessler

Die Dissertation wurde am 10.09.2018 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 13.08.2019 angenommen.

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Characterization of the role of XIAP in modulating inflammatory death and its consequences

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„In der Wissenschaft gleichen wir alle nur den Kindern, die am Rande des Wissens hie und da einen Kiesel aufheben, während sich der weite Ozean des Unbekannten vor unseren Augen erstreckt.“

Sir Isaac Newton

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II. ZUSAMMENFASSUNG

Mutationen im Gen *BIRC4* resultieren in einem Verlust des „X-linked inhibitor of apoptosis“ Proteins (XIAP) und resultieren damit in der Krankheit „X-linked lymphoproliferative disease type 2 (XLP-2)“, eine seltene primäre Immundefizienz. XLP-2 Patienten zeigen eine erhöhte Prädisposition für hämophagozytische Lymphohistiozytose (HLH) und chronisch-entzündliche Darmerkrankung, welche derzeit nur durch eine allogene Stammzelltransplantation aus peripherem Spenderblut geheilt werden kann. Im Hinblick auf aktuelle Publikationen, postulieren wir, dass der Verlust von XIAP in Antigen präsentierenden Zellen (APCs) in einem RIPK3 abhängigen Zelltod resultiert, welcher eine hyperinflammatorische Immunreaktion auslöst und daher für die systematische Deregulation des Immunsystems verantwortlich ist.

Um diese Theorie zu überprüfen wurde ein Kokultursystem mit Toll-like-Rezeptor (TLR) stimulierten dendritischen Zellen zusammen mit T Zellen entwickelt. Dort war es möglich zu beobachten, dass der Zelltod der XIAP defizienten dendritischen Zellen in einer hyperinflammatorischen IL-1β Produktion resultierte und dadurch zu einer starken IL-17 Induktion in $CD4^+$, $\gamma\delta$, peripheren doppelt negative (DN) und iNKT17 T Zellen *in vitro* führte. Diese IL-17 Induktion in angeborenen und adaptiven T Zellsubsets hängt stark von TNF, RIPK3 und Caspase1/8 Signalwegen in dendritischen Zellen ab. Wobei der Verlust von XIAP in T Zellen keine Auswirkung auf die Differenzierung der T Zellen hat.

Intraperitoneale LPS Injektionen in Mäuse verifizierten die *in vitro* Ergebnisse, indem auch hier erhöhte IL-17 Konzentrationen in XIAP defizienten T Zellen gemessen werden konnten welche dann zu einer Rekrutierung von Neutrophilen führte. Außerdem hatten XIAP defiziente Mäuse eine schlechtere Prognose nach Citrobacter rodentium Infektionen, einem Mausmodell welche die Symptome einer chronisch entzündlichen Darmerkrankung von XLP-2 Patienten nachahmt. Ferner waren *Xiap*$^{-/-}$ Mäuse stärker belastet, wenn sie eine Graft-versus-Host Reaktion (GvHR) entwickelten, eine häufige Komplikation von XLP-2 Patienten nach einer Stammzelltransplantation.

Es wurde gezeigt, dass es nach dem Verlust von XIAP zu einem RIPK3 anhängigen Zelltod kommt, welcher in einer erweiterten inflammatorischen Immunantwort resultiert und damit das Gesamtsystem betrifft. Dies zeigt auf, wie wichtig der Zelltod, seine Regulation und die entsprechenden Immunreaktionen für die Balance des Gesamtsystems sind. Ein besseres Verständnis der zu Grunde liegenden Zusammenhänge könnte weitreichende Folgen für die Behandlung von Krankheiten basierend auf inflammatorischem Zelltod haben.

III. ABSTRACT

BIRC4 gene mutations cause deficiency of X-linked inhibitor of apoptosis protein (XIAP) and are therefore the cause of X-linked lymphoproliferative disease type 2 (XLP-2), a rare primary immunodeficiency. XLP-2 patients, who are characterized by elevated susceptibility to hemophagocytic lymphohistiocytosis (HLH) and inflammatory bowel disease (IBD), can only be cured by allogenic peripheral blood stem cell transplantation. Recent findings indicate that loss of XIAP in antigen presenting cells (APCs) results in RIPK3 dependent cell death, which initiates a hyperinflammatory immune response and is therefore responsible for the overall deregulation of the immune system.

To examine the effect of XIAP loss, a coculture system of toll-like receptor (TLR) stimulated WT and *Xiap*$^{-/-}$ dendritic cells and T cells was set up. The death of dendritic cells resulted in the hyperinflammatory production of IL-1β in XIAP deficient DCs and led to a strong IL-17 induction in CD4^{+} T cells, $\gamma\delta$ T cells, double negative T cells and iNKT17 cells in vitro. This IL-17 induction in innate and adaptive T cells subsets strongly depended on TNF, RIP3 and Caspase 1/8 signaling in dendritic cells. Whereas the loss of XIAP in T cells itself had no consequences on their differentiation capability.

Intraperitoneal LPS injections into mice verified the *in vitro* results by showing increased IL-17 in T cells of *Xiap*$^{-/-}$ mice which lead to a recruitment of neutrophils. Moreover, XIAP deficient mice showed a worse prognosis after Citrobacter rodentium infection. The Citrobacter rodentium mouse model mimics the symptoms of the chronic gut inflammation of XLP-2 patients. Lastly, *Xiap*$^{-/-}$ mice displayed a stronger graft versus host (GvHD) reaction after being transplanted, comparable to strong GvHD reactions in XLP-2 patients after allogenic stem cell transplantations.

Summarized, it could be shown that loss of XIAP resulted in RIPK3 dependent inflammatory cell death, followed by an extended inflammatory response involving the overall system. These findings depict once more the importance of cell death regulation and subsequent immune reactions. A better understanding of the underlying correlations might have extensive

consequences regarding therapeutic interventions in diseases with an inflammatory cell death phenotype.

IV. CONTENTS

V. LIST OF FIGURES

VI. ABBREVIATIONS

Instance	Expansion
APC	Allophycocyanin or antigen presenting cell
BM	bone marrow
BMDC	bone marrow derived dendritic cell
BMDM	bone marrow derived macrophage
bp	base pair
BSA	bovine serum albumin
CBA	cytokine bead array
cDNA	complementary deoxyribonucleic acid
CFSE	carboxyfluorescein succinimidyl ester
CpG	TLR-9 receptor anatgonist
CTGLO	cell titer glow
d	day
DMEM	Dulbecco's Modified Eagle Medium
DN	double negative
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DP	double positive
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FADD	Fas-associated protein with death domain
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
fwd	forward
GM-CSF	granolocyte-macrophage colony-stimulating factor

Gsdmd	Gasdermin D
GvHD	graft versus host disease
Gy	gray
h	hour
HBSS	Hanks´ balanced salt solution
HEPES	(*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*)
HLH	hemophagocytic lymphohistiocytosis
H & E	haematoxylin and eosin
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IL	interleukin
IL-1RA	interleukin- 1 receptor antagonist
kDa	kilo Dalton
KO	knockout
LDH	Lactate dehydrogenase
LIV	liver
LN	lymph node
LPS	Lipopolysaccharide
min	minute
miRNA	micro RNA
mJ	milli Joule
ml	milliliter
mRNA	messenger RNA
Nec-1s	Necrostatin-1s
NK	natural killer
nm	nanometer
nM	nanomolar
ns	non significant

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	Phycoerythrin
PI	propidium iodide
pI:C	Poly (I:C)
PLZF	Zinc finger and BTB domain-containing protein 16
RBC	red blood cell
rev	reverse
Ring	real interesting new gene
RIPA	Radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
RT	reverse transcriptase
rpm	rounds per minute
s	second
SDS	sodium dodecyl sulfate
SIRS	systemic inflammatory response syndrome
SN	supernatant
SPL	spleen
TEMED	tetramethylethylendiamin
TCR	T cell receptor
TNF	Tumor necrosis factor
TLR	toll like receptor
VLE	very low endotoxin
WT	wild type
XIAP	X-linked inhibitor of apoptosis
XLP-2	x-linked lymphoproliferative disease type 2

1.Introduction

1.1. X-linked inhibitor of apoptosis

1.1.1.XIAP and its functions

X-chromosome-linked inhibitor of apoptosis (XIAP), encoded by the BIRC4 gene, belongs to the family of inhibitor of apoptosis (IAP) proteins. IAPs were initially identified in baculoviruses (Clem, Fechheimer, and Miller 1991) (Crook, Clem, and Miller 1993). In mammals, the IAP family consists of eight members including XIAP, cIAP1/2, NAIP, ML-IAP, ILP-2, Survivin and Apollon (Salvesen and Duckett 2002).

Linking all proteins to one family is the expression of the unique baculovirus IAP repeat (BIR) domain, which provides the capability for protein interactions (Budhidarmo and Day 2015). XIAP displays three of these BIR domains. In comparison to BIR domains in other IAP proteins, XIAP´s BIRs are the only ones to bind and inhibit caspases -3, -7 and -9. Thereby they have the ability to block induction of extrinsic and intrinsic apoptotic pathways (Deveraux, Takahashi, and Reed 1997; Shiozaki et al. 2003; Scott et al. 2005; Eckelman, Salvesen, and Scott 2006).

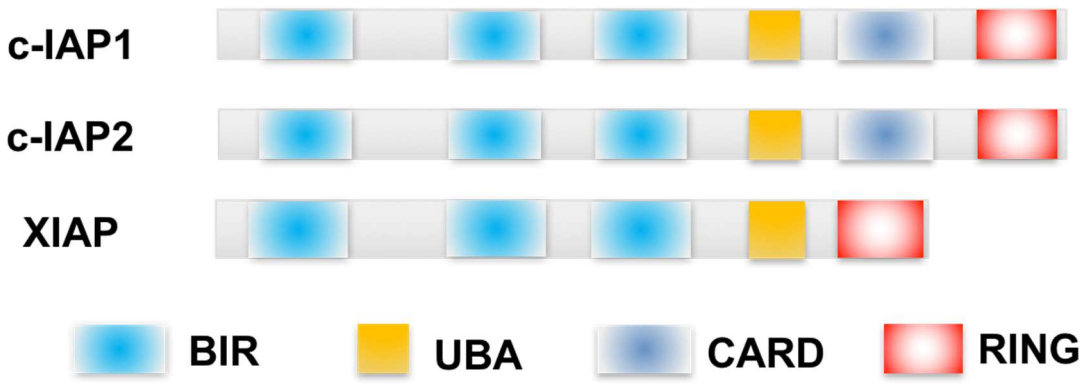


Figure 1. 1.: Protein structure of c-IAP1, c-IAP2 and XIAP.
All three IAPs display three BIR domains, have a ubiquitin binding domain (UBA) and a Ring domain for E3 ligase activity. C-IAP1/2 have an additional CARD domain for protein interactions.

With XIAP´s ability to prevent apoptosis, this protein is often upregulated in cancers, amongst others in childhood de novo AML (Tamm et al. 2004) and breast cancer (Y. Zhang et al. 2011). Therefore, based on the natural IAP antagonist second mitochondrial-derived activator of caspases (Smac) (C. Du et al. 2000), many Smac-mimetics were developed for cancer treatment (Fulda and Vucic 2012).

Recently, knowledge about XIAP´s functionality was broadened after uncovering that the C-terminal RING domain contains E3 ligase activity (Nakatani et al. 2013). With XIAP´s ability to modify proteins with ubiquitin chains, it was discovered that XIAP has a regulatory role in signaling pathways. Examples are activation of NF-κB and MAP kinase (MAPK) downstream of NOD2 (Damgaard et al. 2012) or controlling inflammatory cell death (Yabal et al. 2014; Lawlor et al. 2015). This role in signaling pathways is conveyed through different ubiquitin chains. K11 and K63 linked ubiquitin (U)-chains serve as signaling platforms, while K48 linkage activates proteasomal degradation (Silke and Vucic 2014). Therefore, XIAP can not only build up signaling platforms, but can also specifically block pathways by inducing protein degradation, such as Rac1, which is involved in cell migration (Oberoi et al. 2011), or even XIAP itself through autoubiquitination.

1.1.2.XIAP deficiency and XLP-2 disease

While overexpression of XIAP correlates with poor prognosis in various cancer types, loss of XIAP also has severe consequences. Mutations in *BIRC4* have been associated with X-linked lymphoproliferative syndrome type 2 (XLP-2), a disease first described in 2006 (Rigaud et al. 2006). Mutations in *BIRC4* are mainly located within the BIR2 domain, hit the Ring domain or impair expression of the protein through nonsense or frameshift deletions (Damgaard et al. 2013).

Due to the fact that XIAP is located on the X chromosome, mutations effect 1-2 in 10^6 males (Aguilar and Latour 2015). While heterozygous mothers are usually asymptomatic, first symptoms in affected boys are detectable in early

infancy and often lead to premature death (Rigaud et al. 2006). Most often XLP-2 outbreak correlates with an EBV infection, although other viral infections have been reported as a trigger mechanism, too (Aguilar and Latour 2015). The most frequent clinical manifestations are hemophagocytic lymphohistiocytosis (HLH), splenomegaly and inflammatory bowel disease (IBD) (Latour and Aguilar 2015; Aguilar and Latour 2015). All clinical manifestations can occur together or independently, as for example Crohn´s disease, which has often been reported as the sole clinical manifestation (Speckmann et al. 2013; Yvonne Zeissig et al. 2014). Less frequent are appearances of inflammatory manifestations like uveitis, periodic fever, skin abscesses and Giardia enteritis (Speckmann et al. 2013).

Up to now the immune pathogenesis of XLP-2 is not understood and only a matter of speculations. Though increased T cell death through the Fas receptor was reported *in vitro* (Aguilar and Latour 2015; Rigaud et al. 2006; Speckmann et al. 2013), no T cell lymphopenia is seen in patients (Latour 2007; Veillette, Pérez-Quintero, and Latour 2013).

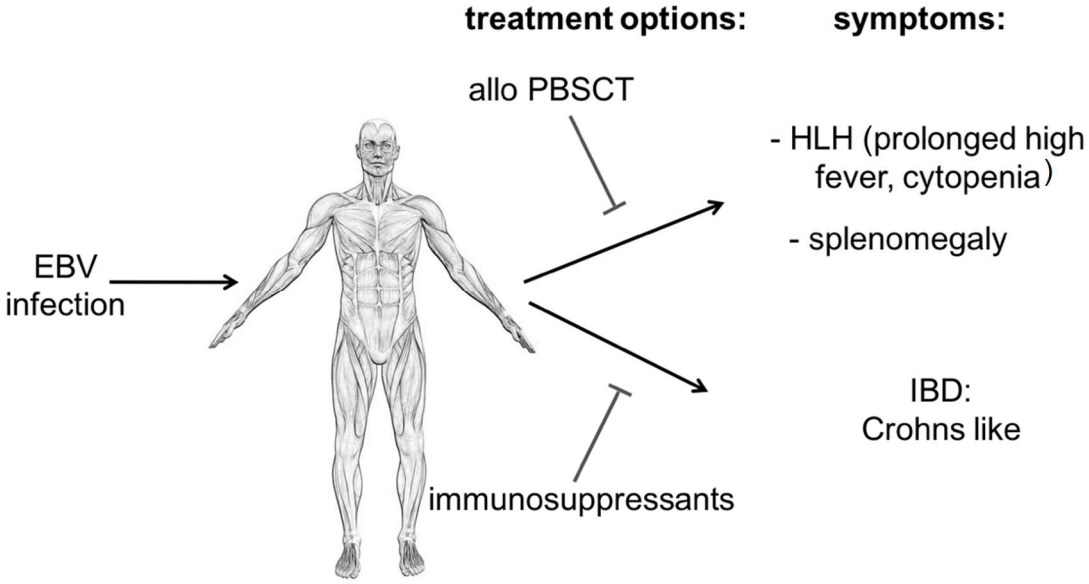


Figure 1. 2.: XLP-2 disease overview

XLP-2 is most often induced by an EBV-infection and results in multiple symptoms with HLH, splenomegaly and IBD being the most common ones. Treatment options can be divided into treating symptoms with immune suppressants or curing the disease with an allogenic peripheral blood stem cell transplantation (allo PBSCT).

One more thing impeding comprehension of the XLP-2 pathology is the fact, that XIAP-deficient mice do not show any signs of impairment or abnormalities without prior stimulation (Harlin et al. 2001; Yabal et al. 2014). Without understanding of the immunopathogenesis, treatment options are limited to dampen either inflammatory symptoms with immunosuppressive treatments or replenish XIAP-deficient stem cells with a peripheral blood stem cell transplantation (PBSCT) (Aguilar and Latour 2015). Unfortunately, a study in 2013 revealed a rather poor prognosis with high lethality after myeloablative conditioning (MAC) and reduced intensity conditioning (RIC) (Marsh et al. 2013). In 2015 this was further confirmed by Latour and Aguilar, who reported a death rate over 20% of XLP-2 patients, with more than 50% dying during allogenic hematopoietic stem cell transplantation (Latour and Aguilar 2015).

1.2. Inflammation and the immune system

1.2.1. From innate to adaptive immunity: PRRs, DCs, cytokines and T helper cells

The immune system of vertebrates consists of two functional domains. The innate immune system responds within minutes to hours and constitutes the first line of defense against invading pathogens. The second domain is the adaptive response which takes days to establish but has the advantage of being more specific and therefore more efficient (Murphy, Travers, and Walport 2008). All cells of the immune system develop from pluripotent hematopoietic stem cells, with most innate immune cells evolving from the myeloid lineage and the lymphoid lineage giving rise to the lymphocytes of the adaptive response (Murphy, Travers, and Walport 2008).

The initial detection of invading pathogens is carried out by pattern recognition receptors (PRRs) of the innate immune response. PRRs can be displayed on the plasma membrane or within organelles, as e.g. Toll-like receptors (TLRs) and C-type lectins. But there are also cytosolic PRRs, e.g. nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) or retinoic acid

inducible gene I (RIG-I) like receptors (RLRs) (Iwasaki and Medzhitov 2010). These receptors recognize conserved patterns, so called pathogen-associated molecular patterns (PAMPs). These can be for instance, lipopolysaccharide (LPS) of gram negative bacteria on TLR4 (Beutler 2009), β-glucans of fungal cell walls by Dectin-1 (Brown 2005), intracellular infections of viral pathogens, like RNA, via RLRs (Yoneyama and Fujita 2010) or bacterial peptidoglycans via NOD1 (Franchi et al. 2009). Moreover, PRR can also detect endogenous molecules from damaged or necroptotic cells called damage-associated molecular patterns (DAMPs) (Matzinger 1994). In case of an infection, DAMP and PAMP release correlates. This results in an elevated, self-fueling activation cycle, as PAMPs can initiate inflammatory cytokines and necroptosis via PRRs, while DAMPs themselves can be recognized by PAMP receptors (Kaczmarek, Vandenabeele, and Krysko 2013).

These PRRs are expressed by nonprofessional immune cells as well as immune cells. One crucial PRR expressing subset comprises of antigen presenting cells (APCs), more precisely dendritic cells and macrophages (Takeuchi and Akira 2010). Within the group of dendritic cells one can distinguish between conventional dendritic cells as well as nonclassical DCs, such as monocyte-derived dendritic cells and plasmacytoid dendritic cells (Mildner and Jung 2014). The classic encounter of a conventional dendritic cell with PAMPs results in three outcomes: pathogen uptake and antigen processing, upregulation of costimulatory molecules and the production of cytokines (Walsh and Mills 2013).

How critical conventional dendritic cells (cDCs) are for proper $CD4^+$ and $CD8^+$ T cell responses, shows the impaired activation of naïve T cells in cDC-lacking mice (Birnberg et al. 2008). T cell activation via antigen presentation by DCs is followed by the progression of naïve T cells into effector subsets. Depending on the encountered cytokines, naïve $CD4^+$ T cells can differentiate into certain effector subsets (Swain 1995) and thereby become producers of inflammatory cytokines like IFNγ (Th1), IL-4 (Th2) or IL-17 (Th17) themselves (Zhu, Yamane, and Paul 2010). To direct specificity of immune responses against pathogen encounters, cDCs produce polarizing cytokines dependent on the pathogen they detect. One example is the differentiation of Th17 cells after

encounter with extracellular bacteria. cDC produced IL-1β, IL-6, TGF- β and IL-23 stimulate Th17 specific T cell differentiation (Walsh and Mills 2013). Differentiated T cell subsets are then capable to mount further immune responses, as for example B cell activation (Murphy, Travers, and Walport 2008).

Although innate and adaptive immunity are two very distinct arms of the immune system, both are crucial for a proper and complete immune response following pathogen encounter and through their entangled teamwork secure pathogen clearance.

1.2.2. Linking innate and adaptive immunity: Innate-like T cells

As just described, T lymphocytes are an important part of the adaptive immune response. Surprisingly, some T lymphocytes cannot be truly counted into the group of adaptive immunity. This subset of so called innate-like T lymphocytes represents a special class in between innate and adaptive immunity. Innate-like T cell lineages are defined by some common features. They are quick responders, serving as sentinels of the immune system. Moreover, they are defined by the expression of semi-invariant αβ or γδ TCRs (Constantinides and Bendelac 2013) and in some cases the dependence on the transcription factor PLZF, in iNKTs, some γδ subsets and mucosal associated invariant T (MAIT) cells (A. K. Savage et al. 2008; Kreslavsky et al. 2009; Emmanuel Martin et al. 2009). According to this description, various T lymphocyte subsets belong to this group of innate-like T lymphocytes and in the following passages three important subsets will be described.

γδ T lymphocytes develop in the thymus from progenitor cells. Most of them then leave the thymus with an already acquired effector function, meaning they are already preprogrammed in their response (Narayan et al. 2012; Muñoz-Ruiz et al. 2017). In contrast to αβ T lymphocytes where somatic rearrangement can result in a multitude of different TCRs, somatic

rearrangement for γδ T cells is rather limited in its diversity (Heilig and Tonegawa 1986). Literature differentiates mouse γδ subsets according to their Vγ chain with Vγ4 and Vγ 6 being predominantly IL-17 γδ T lymphocytes and Vγ1, Vγ5 and Vγ7 producing mostly IFNγ (Papotto, Ribot, and Silva-Santos 2017), therefore linking TCR expression and function (O Brien and Born 2010).

These distinct subsets are not only unique in their expression of cytokines but also migrate to their own anatomical sides throughout the body. Average percentages of γδ T lymphocytes in secondary lymphoid organs vary between 1-5%, but in certain tissues like liver, lung, skin, intestine and reproductive tract, they can constitute up to 50% of T cells (Cheng and Hu 2017) (Carding and Egan 2002). In these mucosal and epithelial tissues γδ T cells function as sentinels and react to pathogen encounter as a first line of defence. Although γδ T cells can recognize conserved non-peptide antigens (Bonneville, O'Brien, and Born 2010; Corpuz et al. 2016), their instant activation is often not bound to TCR engagement. It rather depends on exposure to cytokines like IL-1β and IL-23, which rapidly trigger IL-17 production (Sutton et al. 2009), or is carried out via TLR or Dectin receptor engagement (B. Martin et al. 2009). It was reported, that the strong TCR signal which is important for thymic development is attenuated to acquire innate-like features in the periphery (Wencker et al. 2013).

With their sentinel function γδ T lymphocytes are key to microbial infections, like *M. tuberculosis* (Lockhart, Green, and Flynn 2006)*, C. albicans* (Conti et al. 2014) or *B. pertussis* (Misiak et al. 2016). There, a robust IL-17 production triggers recruitment of neutrophils (Sumaria et al. 2011). Even though γδ T cells are crucial to microbial infections and can also have protective roles in cancer (Silva-Santos, Serre, and Norell 2015), their inflammatory response is not always welcome, as it can result in damage. Therefore γδ T lymphocytes are often associated with autoimmune diseases such as arthritis, colitis, diabetes, psoriasis and EAE (summarized in Papotto, Ribot, and Silva-Santos 2017).

Peripheral DN T lymphocytes got their name from the fact that they do not express the co-receptors CD4 and CD8. They are usually described as being

$CD3^+CD4^-CD8^-\ \alpha\beta^+$, even though some publications also include $\gamma\delta$ T lymphocytes to this group, as $\gamma\delta$ T cells are mainly double negative as well. Up to now there is no specific marker for DN T lymphocytes, making detection rather circuitous with the inclusion of $CD3^+$ and $\alpha\beta$ TCR^+ but the exclusion of $\gamma\delta$ T lymphocytes ($\gamma\delta$ TCR), NKT cells (CD1d-Tetramer binding, NK1.1) and of course $CD4^+$ and $CD8^+$ T lymphocytes.

DN T lymphocytes were reported to be present in low numbers in blood and lymphnodes. Depicting around 1-5% of peripheral blood and lymphoid tissue T lymphocytes in mice and humans (Z. Zhang, Young, and Zhang 2001; Fischer 2005), they constitute a larger subsets in non-lymphoid tissues, among others in lung, kidney, gut epithelium or genital tract (Cowley et al. 2010; Hamad 2010; Johansson and Lycke 2003).

First mentioned 1976 with the *lpr* mouse strain for lupus-like disease, it is known today that the massive accumulation of DN T lymphocytes in this mouse model is due to defective Fas signaling (Martina et al. 2015). Although DN T lymphocytes in the periphery of healthy humans have already been described 1986 (Lanier, J, and H 1986), the origin of these cells remains controversial. The first theory states that DN T cells originate from the thymus, circulate and expand in the periphery (Mixter et al. 1999; Priatel, Utting, and Teh 2001). This is in contrast to a second theory stating that DN T lymphocytes arise from $CD4^+$ or $CD8^+$ T cells after co-receptor downregulation (Dong Zhang et al. 2007; Crispin and Tsokos 2009). This was further supported by a publication showing DN T cell generation after chronic stimulation of $CD4^+$ cells (Grishkan et al. 2013).

According to the available literature, DN T cells are a rather heterogeneous subset with many functions. On the one hand, they have shown important regulatory roles in allogenic transplantation models with increased graft tolerability (Young 2002). Furthermore there were findings, that high DN T lymphocyte counts correlated positively with decreased graft versus host disease (GvHD) induction, due to the ability of DN T cells to inhibit $CD8^+$ pathogenic responses via cytotoxic killing (Young et al. 2003; Young and Zhang 2002). On the other hand, DN T cells are reported to have inflammatory

effects supporting protective immunity against infections like *L. monocyte-genes* (Kadena et al. 2005), *C. leishmenia* (Antonelli et al. 2006), *F. tularensis* (Cowley et al. 2010) and *T. cruzi* (Villani et al. 2010). Moreover DNTs can also have influential roles in autoimmune diseases as in autoimmune lymphoproliferative syndrome (ALPS) (Sneller et al. 1992), systematic lupus erythematosus (SLE) (Crispin et al. 2008), psoriasis (Ueyama et al. 2017) and spondylarthropathy (Sherlock et al. 2012) via IL-17 production.

The last presented innate-like T cell subset are iNKT cells. They constitute the smallest subset of the three of them. Comparable to other innate-like T cell subsets, iNKTs are also represented in higher densities in specific organs, like liver (up to 30% of T cells). Being underrepresented in secondary lymphoid organs such as spleen, lymph nodes or blood of mice (0,5-2,5% of T cells), they are still approx. 10-fold more than in humans (Bendelac, Savage, and Teyton 2007).

Specific for iNKTs is the T cell antigen receptor combination of the V_α-chain $V_\alpha14$-$J_\alpha18$ with the V_β-chain $V_\beta8.2$, $V_\beta7$ or $V_\beta2$ (Bendelac et al. 1994). (Glyco-) lipid based antigens presented via CD1d, a MHC class I-like molecule, are recognized by this semi-invariant TCR (Bendelac 1995) and are sufficient to activate iNKT cells (Kohlgruber et al. 2016). Moreover, iNKTs can be activated like $\gamma\delta$ T cells via cytokines, for example by IL-1β and IL-23, which trigger IL-17 production and secretion (Doisne et al. 2011), or via TLR4 (Kim et al. 2012). Despite similar antigen specificity, iNKT subsets are highly diverse with differences in gene-expression as well as epigenetic controlling, resulting in divergent iNKT subsets with specific effector functions and localizations (Engel et al. 2016). Analogous to Th1, Th2 and Th17, iNKTs can be split into iNKT1, iNKT2 and iNKT17, which produce similar signature cytokines as their adaptive kinship (Brennan, Brigl, and Brenner 2013) and might be one explanation why iNKT are ascribed inflammatory as well as anti-inflammatory attributes.

Development of iNKT cells takes place in the thymus (Pellicci et al. 2002; Watarai et al. 2012) and is controlled through several transcriptional factors, with PLZF being a critical regulator of general iNKT development (Kovalovsky

et al. 2008; A. K. Savage et al. 2008). Other transcription factors further differentiate iNKT subsets into their specific expression profile. One example is RoRγt expression which constitutes a key component for the development of the NKT17 subset (Michel et al. 2008). Its expression consequently results in the production of Th17 cytokines such as IL-17 and IL-22 (Brennan, Brigl, and Brenner 2013).

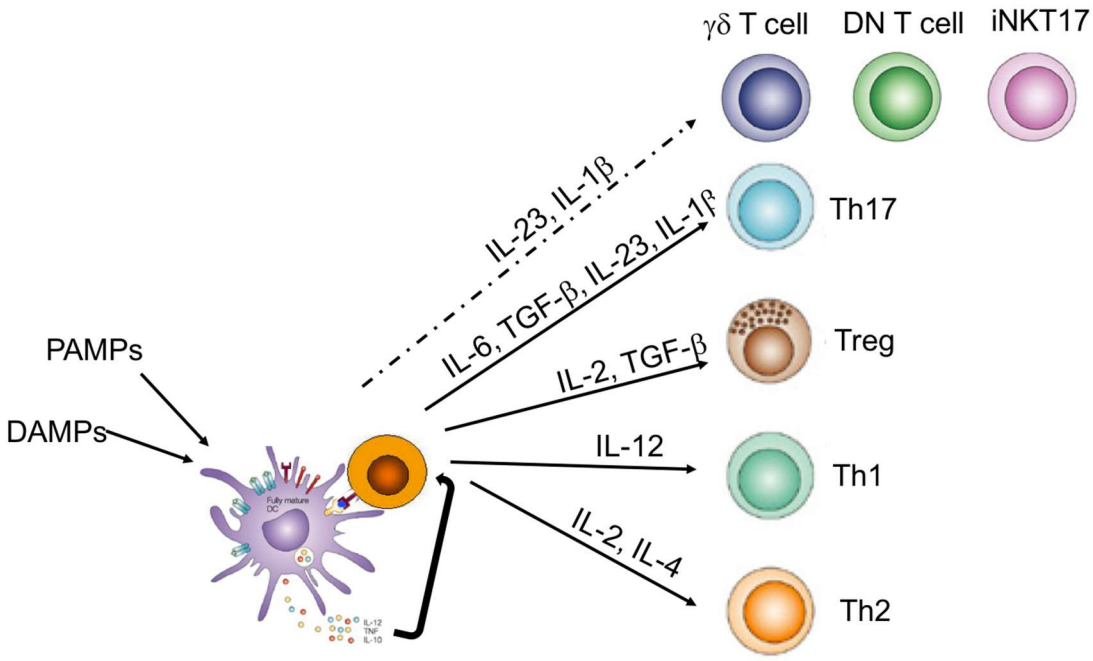


Figure 1. 3.: T cell polarization.

Dependent on the PAMPS and DAMPS an APC encounters, it will produce polarizing cytokines. These cytokines drive then the differentiation in Th17, Treg, Th1 or Th2 cells and activate γδ T cells, DN T cells or iNKT17 cells.

The diversity of iNKT subsets and cytokines also results in diversity of modulating immune responses. This ranges from neutrophil recruitment (Michel et al. 2007), to dendritic cell and macrophage activation (Kitamura et al. 1999; Fujii et al. 2003; NIEUWENHUIS et al. 2002), or B cell modulation (Galli et al. 2007; Hägglöf et al. 2016). Modulating innate as well as adaptive immunity explains the importance of iNKTs in various infections, with *S. pneumonia* (Ranson et al. 2005), *B. burgdorferi* (Kumar et al. 2000) and even influenza virus (Paget et al. 2011) being just a small outtake. Nevertheless, like DN and γδ T cells, also iNKTs are correlated with negative effects in

deregulated excessive immune responses, such as asthma (Pichavant et al. 2008), atherosclerosis (Braun, Covarrubias, and Major 2010) or psoriasis (Peternel and Kaštelan 2009).

1.3. Necroptotic cell death

1.3.1. Death pathways: from controlled implosion to controlled explosion

Cellular processes, like the decision of live and death, are tightly regulated events and are crucial for many processes including development or removal of damaged cells. For the first time, programmed cell death was recognized 1842 by Karl Vogt studying the replacement of the notochord of amphibians (Carl 1842). So, for the longest time of scientific research, the question of cell death revolved about regulated cell death termed apoptosis, autophagy and accidental cell death termed necrosis (Schweichel and Merker 1973).

Apoptosis can be initiated via an extrinsic pathway dependent on death receptors on the cell surface or can be triggered upon intracellular stress. Independent of the triggering mechanism, apoptotic death is always executed by a conserved mechanism relying on caspases, which are synthesized as inactive proteins and can then be activated via dimerization or cleavage (Thornberry and Lazebnik 1998). Apoptosis results in chromatin condensation, contraction of the nucleus and cytoplasm, as well as the cell disintegration into apoptotic bodies. These vesicles are then taken up by phagocytes and due to no membrane rupture, apoptotic cell death does not trigger an immune response (Taylor, Cullen, and Martin 2008).

Only recently other types of cell death were discovered which were initially all summarized under the term necrosis. Up to then, necrosis was the third way of dying without the involvement of phagocytosis and seemed to be “accidental” (Schweichel and Merker 1973). Today it is known that cell death

in forms of pyroptosis, ferroptosis or necroptosis are all very well regulated and are not accidental at all (Tait, Ichim, and Green 2014; Berghe et al. 2014).

Necroptosis is the most well understood pathway of programmed necrosis and on the next pages the execution and consequences of necroptosis will be highlighted.

1.3.2. Prerequisites, induction and execution of necroptosis

Many stimuli that trigger apoptosis can also trigger necroptosis with the premise of caspase inhibition. One critical example for such a caspase would be caspase 8. It is usually involved downstream of death receptors and mediates apoptotic death and therefore blocks necroptosis at the same time (Holler et al. 2000; Yuan, Najafov, and Py 2016). This caspase inhibition can either be virally or synthetically. As mentioned before, IAPs were identified in baculoviruses to inhibit apoptosis and thereby circumventing death. Viruses developed caspase inhibitors to delay cell death allowing to replicate. Thus, necroptosis serves as an antiviral control mechanism in case of caspase inhibition (Cho et al. 2009; Benedict, Norria, and Ware 2002).

The most studied inducers of necroptotic death next to TLRs are death receptor ligands, which induce death via TNF, Fas or TRAIL receptors. This induction is mediated by the “canonical necrosome formation” in contrast to the non-canonical necrosome formation due to dsRNA or under certain circumstance downstream of TLR3 and TLR4. Independent of the mechanism triggering necroptosis, key executioner molecules are RIPK3 and MLKL. The activating factor of RIPK3 discriminates the canonical and non-canonical pathway, but both depend on the interaction with the RIPK homology interaction motif (RHIM) domain expressed by RIPK3 (Oberst 2015). In the canonical pathway RIPK1 exhibits this RHIM domain as well and interacts through it with RIPK3 in a kinase dependent manner for activation (He et al. 2009). In the non-canonical context, DAI (in a viral setting) and TRIF (downstream of TLR3/4) can activate RIPK3 via their own RHIM domains (Upton, Kaiser, and Mocarski 2012; Kaiser et al. 2013). More recently even

signaling via interferon receptors was discovered to be an inducer of necroptosis (Roshan J Thapaa et al. 2013). Ultimately, whatever the initiating mechanism, activation of RIPK3 triggers the translocation of MLKL into the plasma membrane and thereby the execution of necroptotic death (H. Wang et al. 2014).

1.3.3. Necroptosis downstream of the TNF receptor

The most well studied necroptotic pathway is downstream of the TNF receptor (Figure 1.4). Upon binding of TNF to the TNF receptor the assembly of the receptor bound complex I can be detected, which has pro-survival and pro-inflammatory implications.

After ligation of TNFR1 by TNF, RIPK1 and the adaptor protein TRADD are recruited to the receptor (Zheng et al. 2006). This is followed by binding of TRAF2 (Hsu et al. 1996) and the subsequent relocation of the ubiquitin ligases cIAP1/2 to the complex (Vince et al. 2009; Mace et al. 2010). Amongst others, cIAP1/2 then ubiquitinate RIPK1 and themselves with K11 and K63 linked ubiquitin-chains and these chains then serve as scaffolds to recruit further proteins (Park, Yoon, and Lee 2004; Varfolomeev et al. 2008). Recruited proteins contain the TAB/TAK complex, the IKK complex and LUBAC. Additional linear M1 linked ubiquitin chains attached from LUBAC amplify the downstream signaling events including MAPK and NF-κB signaling for survival and inflammation (Dondelinger et al. 2016).

The stability of complex I depends on deubiquitinating enzymes like CYLD that destabilize complex I through the deubiquitination of RIPK1 (Andrew Kovalenko et al. 2003). This allows RIPK1 and TRADD to dissociate from the TNF receptor and therefore the plasma membrane. In addition, blocked NF-κB signaling or perturbed ubiquitination can lead to the formation of complex IIa or IIb respectively. Complex IIa is based on TRADD binding to FADD and recruiting Caspase 8, while complex IIb is based on RIPK1 binding to FADD and recruitment of Caspase 8 (Dondelinger et al. 2016). Active Caspase 8 cleaves RIPK1 and RIPK3 preventing necroptosis and initiating apoptosis (Lin

et al. 1999; Feng et al. 2007). As mentioned above, in case of caspase inhibition necroptosis can be induced, which is also the case downstream of TNF receptor signaling. Under caspase inhibiting conditions the necrosome assembles including RIPK1, RIPK3 and MLKL. RIPK1 thereby recruits RIPK3 via their RHIM domains and through oligomerization and phosphorylation RIPK3 is activated (Li et al. 2012; Cho et al. 2009; He et al. 2009). Recruitment and phosphorylation of MLKL eventually leads to the execution of necroptotic death with the translocation and integration of MLKL into the plasma membrane causing pore formation (H. Wang et al. 2014).

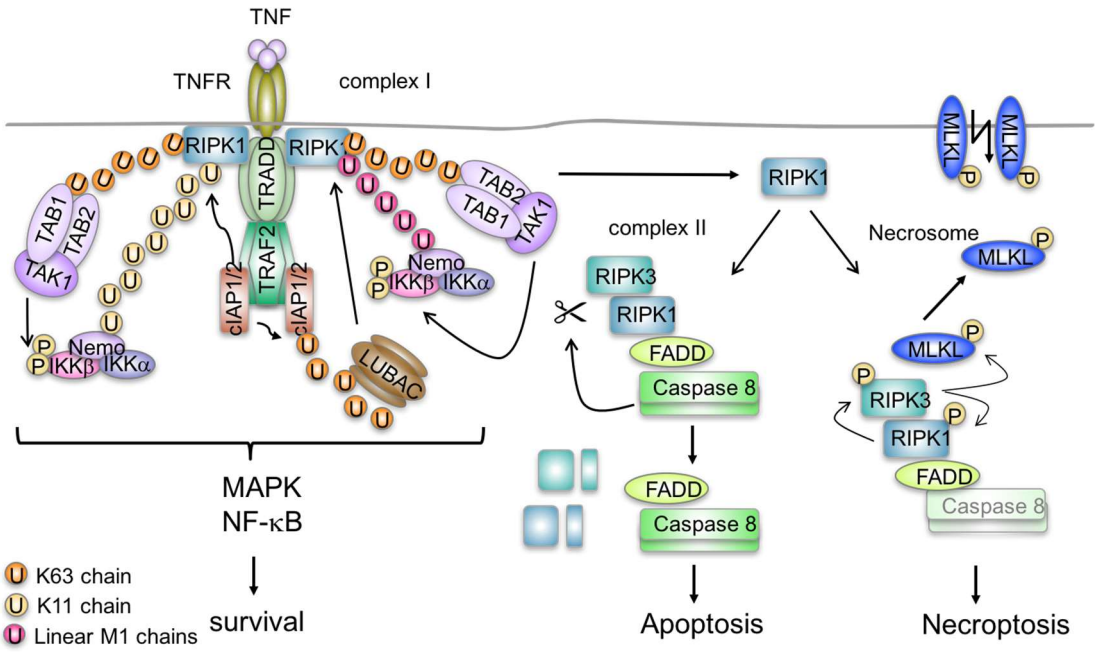


Figure 1. 4.: Signaling events downstream of the TNF receptor.
Binding of TNF to its receptor triggers the formation of complex I and results in the induction of survival genes via MAPK and NF-κB signaling. Under certain circumstances RIPK1 can dissociate from the receptor and thereby trigger apoptosis via Caspase 8. In case of caspase inhibition assembly of the necrosome results in necroptotic death.

1.3.4. Necroptosis: its consequences and clinical implications

As summarized in the chapters before necroptosis is, like apoptosis, a strictly regulated form of cell death but nonetheless it shares similarities with necrosis in the consensus of being inflammatory. This inflammatory property is due to the particular execution of necroptotic cell death. In contrast to apoptosis with its packaging of cellular contents into apoptotic bodies, necroptosis is like an “ordered cellular explosion” (Vandenabeele et al. 2010). With plasma membrane rupturing and spilling of cellular contents into extracellular space, necroptosis can be distinguished from apoptotic death by means of morphological changes, membrane permeability detected by flow cytometry and detection of intracellular contents (Krysko et al. 2008).

Endogenous cellular contents can be damage associated molecular patterns (DAMPs) like DNA, RNA, ATP. Also chromatin associated proteins such as HMBG1 (Kaczmarek, Vandenabeele, and Krysko 2013), or inflammatory molecules, termed alarmins, such as IL-1 family cytokines can serve as DAMPs (S. J. Martin 2016). As described before in chapter 1.2.1 DAMPs can be recognized by PRRs and work together with PAMPs to fuel inflammatory processes.

Therefore, necroptotic death is most often not linked to embryonic development or the regulation of cell populations, processes clearly regulated by apoptosis, but is rather associated with disease (Grootjans, Berghe, and Vandenabeele 2017). Involvement of necroptosis could be linked to several highly inflammatory conditions, such as systemic inflammatory response syndrome (SIRS) (Duprez et al. 2011) and ischemic reperfusion injury (Linkermann et al. 2012) as well as chronic and autoimmune diseases like inflammatory bowel disease (IBD) (Pierdomenico et al. 2013) or multiple sclerosis (Ofengeim et al. 2015).

1.4. Research Objective

XLP-2 disease correlates with high mortality and affects patients at a very young age. Patients suffer from various symptoms like HLH, splenomegaly, IBD and other inflammatory manifestations. So far, the disease pathology is not understood. This limits treatment options to immunosuppressant therapy or peripheral stem cell transplantation, which correlates with high mortality rates likewise (Marsh et al. 2013).

XLP-2 disease is caused by a mutation in the XIAP encoding gene *BIRC4*. XIAP was originally discovered for its caspase inhibiting properties. However, research in the last years has revealed a function for XIAP and some of its family members in signal transduction. This function is dependent on the E3-ligase Ring domain, which equips proteins with the ability to modify targets with ubiquitin chains to allow for signal transduction or degradation.

A recent lab own publication discovered that in the context of XIAP deficiency, or loss of its E3-ligase function, antigen presenting cells are sensitive to a TNF driven RIPK3-dependent inflammatory death (Yabal et al. 2014). As XLP-2 symptoms are in summary described as a hyperinflammatory overreaction of the immune system, it is hypothesized that the reason therefore might be lying in XIAP driven inflammatory cell death which strongly drives further immune reactions and thereby starts a cycle of inflammation.

Thus, the aim of this thesis was to elucidate the consequences of XIAP driven inflammatory cell death on the surrounding environment and how it is regulated. Getting a deeper understanding of the signaling events and their consequences, will not only help improve treatment options for XLP-2 patients, but will also guarantee a better understanding of necroptosis and its subsequent immune modulatory functions in other diseases.

2. Material

2.1. Reagents

If not stated otherwise, all chemicals were purchased from Sigma-Aldrich. Additional reagent and kit information is provided in the respective methods section.

2.2. Antibodies

2.2.1. For Western blotting

Antibodies were used in the dilution recommended by manufacturer.

Identification	Clone	Species	Company
αGsdmd (hs)	G7422	rabbit	Sigma
αCaspase 8 (mm)	1G12	rat	Gift
αIL-1β (mm)	AB-401-NA	goat	R&D Systems
αACTIN, HRP tagged (mm)	13E5	rabbit	Cell Signaling
αRabbit IgG, HRP tagged	polyclonal	goat	Jackson IR
αRat IgG, HRP tagged	polyclonal	goat	Jackson IR
αgoat IgG, HRP tagged	polyclonal	goat	Jackson IR

2.2.2.For FACS analysis and sorting

All antibodies were used in a 1:400 dilution.

Identification	conjugate	clone	Company
αCD4	PeCy5	RM4-5	eBioscience
αCD4	APC	GK1.5	eBioscience
αCD8a	APC	53.6-7	eBioscience
αCD3ε	FITC	145-2C11	eBioscience
αγδTCR	PE	eBioGL3	eBioscience
αIL-17	Brilliant Violet	TC11-18H10.1	BioLegend
αIL-17	PeCy7	eBio17B7	eBioscience
αIFNγ	PeCy7	XMG1.2	eBioscience
αIFNγ	PE	4S-B3	eBioscience
αRoRγt	PerCPeFluor710	B2D	eBioscience
αPLZF	Alexa Fluor488	Mags.21F7	eBioscience
αFoxP3	FITC	FJK-16s	eBioscience
αCD11b	PeCy5	M1/70	eBioscience
αCD11b	APC-Cy7	M1/70	eBioscience
αCD11c	APC	N418	eBioscience
αCD11c	FITC	N418	eBioscience
αTCRβ	FITC	H57-597	eBioscience
αNK1.1	FITC	PK136	eBioscience
αMHC 1 (H-2kb)	APC	AF6-88.5.5.3	eBioscience
αMHC I (H-2kd)	PE	SF1-1.1.1	eBioscience
αCD16/32	purified	93	eBioscience
αCD25	PE	PC61.5	eBioscience

αCD80	PE	16-10A1	eBioscience
αCD86	PE	GL1	eBioscience
αCD103	APC	M290	BD Bioscience
αLy-6B.2	APC	7/4	Bio-Rad
αMHC II	FITC	M5/114.15.2	eBioscience
αF4/80	APC-Cy7	BM8	eBioscience
αLy-6G (Gr-1)	eFluor 450	HK1.4	eBioscience
αmCD1d Tetramer	APC	---	Nat. Inst. Of Health Tetramer Core Facility

2.3. Employed primers

Primers were synthesized at Sigma-Aldrich or MWG-biotech.

2.3.1. Genotyping primers

Identification	**sequence 5´> 3`**
XIAP WT fwd	GTAGGCAGGAGGTTTAGAAG
XIAP neo fwd	TCCTCGTGCTTTTCGGTATC
XIAP rev	GATTCCTCAAGTGAATGGGT
XIAP ΔRing fwd	TAAAGCCTTTACCTTCTTCTCTATTT
XIAP ΔRing rev	TGGGACAGGTAGGATTTAGTGCTTCG
TNF fwd	TAG CCA GGA GGG AGA ACA GA
TNF WT rev	AGT GCC TCT TCT GCC AGT TC
TNF ko rev	CGT TGG CTA CCC GTG ATA TT
RIPK3 1	CGCTTTAGAAGCCTTCAGGTTGAC
RIPK3 2	GCCTGCCCATCAGCAACTC
RIPK3 3	CCAGAGGCCACTTGTGTAGCG
MLKL 1	TAT GAC CAT GGC AAC TCACG
MLKL 2	ACC ATC TCC CCA AAC TGT GA

MLKL 3	TCC TTC CAG CAC CTC GTAAT
Caspase 1 WT fwd	GAG ACA TAT AAG GGA GAA GGG
Caspase 1 ko fwd	TGC TAA AGC GCA TGC TCC AGA CTG
Caspase 1 rev	ATG GCA CAC CAC AGA TAT CGG
IL1r1 fwd	CTCGTGCTTTACGGTATCGC
IL1r1 WT	GGTGCAACTTCATAGAGAGATGA
IL1r1 rev	TTCTGTGCATGCTGGAAAAC

2.3.2.Real time primers

Identification	**sequence 5´> 3`**
Actin fwd	AAGAGCTATGAGCTGCCTGA
Actin rev	TACGGATGTCAACGTCACAC
Pro Il-1β fwd	TGTAATGAAAGACGGCACACC
Pro IL-1β rev	TCTTCTTTGGGTATTGCTTGG
Tnf fwd	CCACGCTCTTCTGTCTACTGAAC
Tnf rev	TTGTCACTCGAATTTTGAGAAGATG
IL-6 fwd	CAGGATACCACTCCCAACAGACC
IL-6 rev	AAGTGCATCATCGTTGTTCATACA
IL-23p40 fwd	GGAAGCACGGCAGCAGAATA
IL-23p40 rev	AACTTGAGGGAGAAGTAGGAATGG

3. Methods

3.1. Work with dendritic cells and macrophages

3.1.1. BMDC differentiation

VLE RPMI was supplemented with 10% *Sera Plus* fetal bovine serum (PAN Biotech GmbH), 1% Glutamin (Gibco), 1% PenStrep (Gibco), 0,1% ß-Mercaptoethanol (Gibco) and with 10ng/ml GM-CSF (Peprotech) for the differentiation of BMDCs. Mice were sacrificed and tibia and femur were extracted under sterile conditions. The bone marrow was flushed out with media and erythrocytes were eliminated with G-DEX^{TM} II RBC lysis buffer (iNtRON Biotechnology). Cell numbers were determined in a Neubauer Counting Chamber (Omnilab) in 0.2% Trypan Blue (Life Technologies) and $5x10^6$ bone marrow cells were seeded per 10cm uncoated cell culture dish with 10ml GM-CSF supplemented RPMI media as described before. One day three additional 10ml media was added. One day six 10ml were retrieved from the dish, centrifuged and replaced with fresh GM-CSF media. On day seven supernatant containing differentiated and unstimulated BMDCs were collected and loosely attached BMDCs were harvested by rinsing with PBS. BMDCs were pelleted and resuspended in GM-CSF free RPMI (Gibco) supplemented with 10% fetal bovine serum (Capricorn), 1% Glutamin, 1% PenStrep, 0,1% ß-Mercaptoethanol.

3.1.2. BMDM differentiation

For BMDM differentiation L929 enriched media is needed. Therefore, L929 cells were seeded in 20cm coated dishes with 30-40ml media containing RPMI (Gibco) supplemented with 10% fetal bovine serum (Capricorn), 1% Glutamin, 1% PenStrep, 0,1% ß-Mercaptoethanol. After 7-9 days media containing M-CSF was harvested and filtered sterile before usage. Bone marrow cells from femur and tibia of mice with respective genotype were seeded in RPMI media containing 20% L929 enriched media. On the following day only the floating

non-differentiated cells were harvested and reseeded at a density of $5x10^6$ cells per 10cm uncoated dish, again in 20% L929 media containing media. By day five most cells should have attached so that media can be easily replaced with fresh media containing 10% L929 enriched media. On day 7 cells were harvested and used for experiments in RPMI media not containing L929 media.

3.1.3. Analysis of differentiation markers

For differentiation verification and BMDC characterization, cells were seeded in uncoated dishes (Greiner bio one) overnight and treated with 5ng/ml LPS (Invivogen, ultrapure). After 24hours cells were harvested with PBS-EDTA (2mM) and Fc receptors were blocked on ice in PBS together with a LIVE/DEAD Fixable Near-IR Dead Cell stain kit (life technologies). After 10min, stains for BMDC surface markers (CD11c, CD11b, Gr-1, 7/4, MHCII, F4/80) were added for an additional 20min. Flow cytometric analysis was performed on a FACS Canto II (BD) and data was analyzed with FlowJo software.

3.1.4. Protein analysis

To examine protein expression of BMDCs, cells were seeded in Optimem media (Gibco) and treated for 8h with LPS (Invivogen, ultrapure) or for 2h with LPS and additional 30min with 5mM ATP (Invivogen) as a control. After indicated time points, floating and attached cells were lysed with a cytosolic fraction enriching buffer (20mM HEPES, 1% NP40, 5mM NaCl, 140mM KCl, 1mM DTT, 1x protease inhibitor cocktail). Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and 30μg per sample was loaded on a 12% SDS gel and afterwards transferred on a nitrocellulose membrane (Omnilab). Membranes were incubated with indicated antibodies over night at 4°C and after secondary antibody exposure, protein expression was detected with a Western Blot Imager (Intas).

3.1.5. Quantitative real-time PCR

To examine mRNA expression of dendritic cells, BMDCs were treated with LPS 5ng/ml for 24 hours. RNA was obtained from treated cells with NucleoSpin® RNA II (Macherey-Nagel) according to manufacturer's instruction and concentrations were quantified with a Nano Drop 2000 (Thermo Scientific). 1μg per sample was used for cDNA synthesis with Reverse Transcriptase II (Invitrogen according to manufacturer's instruction) and was thereafter diluted 1:10 with nuclease-free water (Promega) for real-time PCR quantification. According to manufacturer's instruction quantitative RT-PCR was performed with 2,5μl cDNA per sample and listed primers (Section 3.3.2) using the GoTaq qPCR Mastermix (Promega) and the LightCycler 480 II (Roche).

3.1.6. Treatments

For coculture setups day 7 BMDCs were seeded in U-bottom 96 well plates and for cytokine detection and viability assays BMDCs were seeded in F-bottom plates. Independent of experimental fate $2{,}5 \times 10^5$ BMDCs per well were seeded und treated with the following stimuli: LPS 2ng/ml (Invivogen, ultrapure) CpG ODN 10nM (Invivogen), pI:C 10μg/ml (Invivogen), Anakinra 15μg/ml (Swedish Orphan Biovitrum AB), recombinant mouse TNF 100ng/ml (Biolegend), recombinant mouse IL-1β 1ng/ml (Peprotech), Z-IETD-FMK 10μM (BD Biosciences), Necrostatin-1s 30μM (R&D Systems), GSK1728A 10μM (GSK). Inhibitor used in combination with a TLR antagonist were added 45min before TLR stimulation. UVC irradiation was applied in doses of 5,10 or 20mJ/cm^2 with a CL-1000 Ultraviolet Crosslinker (UVP).

3.1.7. Viability assays

After 24 hours of treatments (Section 4.1.6) viability and survival of dendritic cells or macrophages was assessed in three ways. First, viability of BMDCs and BMDMs was detected by measurement of ATP. This was quantified with Cell Titer Glo Luminescent Cell Viability Assay (Promega) according to manufacturer´s recommendation.

To detect lytic cell death of BMDCs, LDH release was quantified with a CytoTox 96 Non-Radio. Cytotoxicity Assay (Promega) after the company´s instruction.

To verify increased death rate in BMDCs with a third available option, 5μM propidium iodide (Sigma-Aldrich) was added to cells for the last 30minutes of treatment and transmitted light pictures as well as PE Texas Red fluorescence pictures were taken (EVOS FL from life technologies).

3.1.8. Cytokine quantification

After 24 hours, supernatant of treated cells was collected, diluted if necessary and cytokines were measured with a Cytokine Bead Array (for TNF, IL-6 and IL-1β) (BD Biosciences) or ELISA (for IL-23) (Biolegend) according to manufacturer's instruction.

3.2. Work with T lymphocytes

3.2.1. Purifications of T lymphocytes

To purify CD4 and γδ T lymphocytes for a coculture setup as well as naïve CD4 lymphocytes, spleen and superficial cervical, axillary, inguinal and mesenteric lymphnodes from WT Bl6, *Xiap*$^{-/-}$ Bl6 or WT Balb/c mice were collected and meshed through a 100μm strainer. After red blood cell lysis (with G-DEX™ II RBC lysis buffer (iNtRON Biotechnology)), cells were labeled with antibodies and beads according to manufacturer´s description and were

subject to purification via MACS bead (Milteny Biotec, negative selection $CD4^+$ T cell isolation Kit II for coculture and positive selection for naïve $CD4^+$ T cell isolation Kit for differentiation setups). Purity was verified with a simple FACS analysis for CD4 and γδ T cells.

For purification of either CD4, γδ or double negative T lymphocytes from WT Bl6 mice, single cell suspension of spleen and mentioned lymph nodes was prepared as described before. Cells were then subject to staining (CD3, CD4, CD8, γδ TCR) and were sorted into highly pure subsets of $CD3^+CD4^+$, $CD3^+$ γδ TCR^+ or $CD3^+$ γδ TCR^- $CD4^-$ $CD8^-$ with a FACS Aria III.

3.2.2. Coculture setup and analysis

For coculture setup MACS purified or sorted T lymphocytes were washed, counted and resuspended in RPMI (Gibco) supplemented with 10% fetal bovine serum (Capricorn), 1% Glutamin, 1% PenStrep, 0,1% ß-Mercaptoethanol and seeded onto 24 hours stimulated BMDCs. When proliferation assessment was wanted, purified T lymphocytes were subject to CFSE (eBioscience) staining according to instructions from manufacturer before addition of stained T cells to coculture. Proliferation of T lymphocytes was then traceable in the FACS via detection on the FITC channel. Coculture was maintained for 5 days in a sterile cell culture incubator (Binder).

After 5 days of coculture T lymphocytes were restimulated with a mix of PMA (0,5nM) / Ionomycin (0,1nM) / Brefeldin A (Biolegend 5ng/ml) for 4 hours and were then pipetted out of the 96 well plate, were washed, Fc receptor was blocked and cells stained with fluorescent labeled antibodies for viability, CD4, γδ TCR, CD1d-Tetramer, CD3ε, TCRβ, CD8a, NK1.1. Intracellular staining of IL-17, IFNγ, RoRγt and PLZF was performed with the FoxP3 Staining Buffer Set (eBioscience) according to protocol overnight. Moreover, IL-17 concentrations in the supernatant of coculture was measured by ELISA (R&D Systems).

3.2.3.*In vitro* differentiation of T cells

Naïve CD4$^+$ CD62L T lymphocytes from spleen and lymphnodes of WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring}$ mice were purified like described before (Section 4.2.1.) with the naïve CD4$^+$ T cell Isolation Kit (Milteny Biotec) and were seeded into overnight pre-coated rabbit anti Syrian Hamster Ig (H+L) (10μg/ml, Dianova) and one hour coated anti mouse CD3 (5μg/ml, eBioscience) tissue culture plates. T Lymphocytes were seeded into RPMI (Gibco) supplemented with 10% fetal bovine serum (Capricorn), 1% Glutamin, 1% PenStrep, 0,1% ß-Mercaptoethanol and supplemented with anti-mouse CD28 (2,5μg/ml, eBioscience). According to the specific differentiation protocols, additional cytokines and antibodies were added:

- Treg differentiation: 4ng/ml recombinant human TGFβ (R&D Systems)
  5ng/ml recombinant mouse IL-2 (R&D Systems)
- Th17 differentiation: 4ng/ml recombinant human TGFβ (R&D Systems)
  10ng/ml IL-6 (R&D Systems)
  10μg/ml anti IFNγ (eBioscience)
  5μg/ml anti IL-4 (eBioscience)
  5μg/ml anti IL-2 (eBioscience)
- Th1 differentiation: 5ng/ml IL-12 (R&D Systems)
  5μg/ml anti IL-4 (eBioscience)
  5ng/ml recombinant mouse IL-2 (R&D Systems)

T lymphocytes were stimulated for three days and were then subject to flow cytometric analysis for CD4, CD25 and intracellular expression of either FoxP3, IL-17 or IFNγ.

3.3. Work with mice

3.3.1. Husbandry and breeding

All animals were housed under standardized, specific pathogen free conditions in individually ventilated cages (TechniPlast IVC). Studies were conducted in compliance to federal and institutional guidelines. Animal protocols were approved by the government of Oberbayern.

Xiap$^{-/-}$ (Olayioye et al., 2005) and *Xiap*$^{\Delta RING/\Delta RING}$ (Schile et al., 2008) mice have been previously described. *Tnf*^{tm1Gkl} (*Tnf*$^{-/-}$) mice (Pasparakis et al., 1996) were purchased from Jackson Laboratories. *Rip3*$^{-/-}$ (NeWTon et al., 2004) mice were obtained under a material transfer agreement from Genentech and have been previously described. *Caspase 1/11*$^{-/-}$ (K. Kuida et al., 1995) mice were a kind gift from Olaf Gross. *Mlkl*$^{-/-}$ mice were described before (Murphy et al, 2013). All mice were backcrossed into the C57BL/6 background. Both male and female mice deficient for the XIAP gene *BIRC4* were denoted *Xiap*$^{-/-}$. Interbreeding the *Xiap*$^{-/-}$ and *Tnf*$^{-/-}$ generated the XIAP and TNF double deficient mice *Xiap*$^{-/-}$*Tnf*$^{-/-}$, interbreeding the *Xiap*$^{-/-}$ and *Rip3*$^{-/-}$ generated double deficient *Xiap*$^{-/-}$*Rip3*$^{-/-}$ mice and interbreeding *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ generated double deficient *Xiap*$^{-/-}$ *Caspase1/11*$^{-/-}$ mice and interbreeding *Xiap*$^{-/-}$ and *Mlkl*$^{-/-}$ mice generated double deficient *Xiap*$^{-/-}$*Mlkl*$^{-/-}$ mice. All mice were treated and held according to the ethics commitee of the Klinikum rechts der Isar and approved by the government of Oberbayern.

3.3.2. Genotyping

For genotyping ear hole punches were used for DNA extraction with the Wizard SV Genomic DNA Purification System (Promega) according to protocol and PCR setups were performed with 19µl of the GoTaq Green Master Mix

(Promega) and 1μl of purified DNA and run on PCR cyclers (Bio–Rad) according to protocol.

Gene name	PCR protocol	band size
Xiap$^{-/-}$	1: 94°C for 4min; 2: 94°C for 40sec; 3: 55°C for 30sec 4: 72°C for 60sec; 5: Back to 2/ for 30 cycles 6: 72°C for 5min; 7: 4°C hold	WT 500bp ko 600bp
Xiap $^{\Delta Ring}$	1: 94°C for 4min; 2: 94°C for 40sec; 3: 55°C for 30sec 4: 72°C for 60sec; 5: Back to 2/ for 30 cycles 6: 72°C for 5min; 7: 4°C hold	WT 150bp ko 200bp
Tnf$^{-/-}$	1: 94°C for 3min; 2: 94°C for 30sec; 3: 62°C for 1min 4: 72°C for 1min; 5: Back to 2/ for 35 cycles 6: 72°C for 2min; 7: 4°C hold	WT 183bp ko 318bp
Mlkl$^{-/-}$	1: 96°C for 2min; 2: 96°C for 30sec; 3: 57°C for 30sec 4: 72°C for 1min; 5: Back to 2/ for 30 cycles 6: 72°C for 5min; 7: 4°C hold	WT 498bp ko 158bp
Caspase1$^{-/-}$	1: 94°C for 2min; 2: 94°C for 20sec; 3: 65°C for 15sec (*–0.5°C per cycle decrease); 4: 68°C for 10sec 5: repeat steps 2-4 for 10 cycles; 6: 94°C for 15sec 7: 60°C for 15sec; 8: 72°C for 10sec 9: repeat steps 6-8 for 28 cycles; 10: 72°C for 2min 11: 4°C hold	WT 500bp ko 300bp
Ripk3$^{-/-}$	1: 94°C for min; 2: 94°C for 1 min; 3: 60°C for 30sec 4: 72°C for 1min; 5: Back to 2/ for 30 cycles 6: 72°C for 10min; 7: 4°C hold	WT 320bp ko 485bp
IL1r1$^{-/-}$	1: 94°C for 2min; 2: 94°C for 20sec; 3: 65°C for 15sec (*-0.5°C per cycle decrease); 4: 68°C for 10sec 5: repeat steps 2-4 for 10 cycles; 6: 94°C for 15sec 7: 60°C for 15sec; 8: 72°C for 10sec 9: repeat steps 6-8 for 28 cycles; 10: 72°C for 2min 11: 4°C hold	WT 310bp ko 150bp

After PCR reaction, samples were loaded onto a 2% agarose gel and PCR products were separated via electrophoresis.

3.3.3. Intraperitoneal LPS injections

For intraperitoneal LPS injections mice (both male and female littermates) of 8-12 weeks´ age were used. 200μg of LPS (ultrapure, Invivogen) in 200μl of PBS was injected into the peritoneum of each mouse and peritoneal fluid and serum were collected either after 4 hours or 24 hours. For cytokine measurements, peritoneal fluid was concentrated with 10kDa MWCO Vivaspin filters (Vivascience) and then quantified together with serum with the enhanced Cytokine Bead Array (BD Bioscience) according to manufacturer´s instruction. For T lymphocyte analysis, cells from spleen, draining lymphnodes and peritoneal fluid were collected, processed into single cell suspensions, subject to red blood cell lysis (with G-DEX™ II RBC lysis buffer (iNtRON Biotechnology)) and restimulation (according to description in Section 3.2.2). After Fc blocking, a LIVE/DEAD Fixable Near-IR Dead Cell stain kit (life technologies) was used according to protocol for exclusion of dead cells, followed by staining for CD3, CD4, CD8, γδ TCR, Cd1d-Tetramer, Gr-1, Ly6B, CD11b and intracellular IL-17 with the FoxP3 Staining Buffer (eBioscience) over-night and flow cytometric analysis following the next day.

3.3.4. Citrobacter rodentium

To induce colitis in WT and *Xiap*$^{-/-}$ Bl6 mice, male and female littermates of 8-12 weeks of age were cohoused for at least 5 weeks. Inoculation of mice was done by oral gavage of 4-8x10^9 CFU of Citrobacter rodentium strain ICC 169, with bioluminescence, after 8 hours of starvation. Every day, the mice were subject to weight control and detection of bacterial burden by bioluminescence imaging with an IVIS Lumina II system. To simplify detection of bioluminescence, the bellies of the mice were shaved before. After 14 days, when bioluminescence was not or almost non-detectable, mice were sacrificed and the distal colon was washed and fixed with 4% PFA over night for histological analysis (Section 3.3.6.4.). 3μm-thick sections were cut, deparaffinized and stained with hematoxylin and eosin (H&E) according to standard protocols.

To further analyze differences between genotypes, mice were sacrificed at day seven, the peak of the Citrobacter rodentium infection and cells in the lamina propria and intraepithelial compartment from ileum and colon were purified and analyzed as described in Section 3.3.6.3. and FACS analyzed for CD3, γδ TCR, CD4, IL-17, IL-22.

3.3.5. Graft versus host

3.3.5.1. Induction and engraftment verification

For GvHD induction in Bl6 mice, male and female littermates of WT and *Xiap*$^{-/-}$ mice were cohoused prior to experiments for at least 5 weeks. After optimization of GvHD induction protocols, mice were given 0,015% Borgal (Virbac) with 150mg Aspartam (Fagron) into their drinking water two days before GvHD induction and were kept on antibiotics for the first ten days after irradiation. For induction of GvHD in bought, clean Balb/c mice, this was not necessary. To induce GvHD, recipient mice were irradiated with a lethal split dose of 11Gy for Bl6 or 9Gy for Balb/c mice 4-6 hours apart with a Gulmay irradiation unit. After the second round of irradiation, mice were transplanted. To induce GvHD in mice, transplantation of bone marrow mixed with CD4 and CD8 T lymphocytes is necessary. Therefore, bone marrow and spleen was obtained from donors and treated with G-DEX^{TM} II RBC lysis buffer (iNtRON Biotechnology). CD4 and CD8 T lymphocytes were purified from spleens according to protocol for CD4 (L3T4) and CD8a (Ly-2) MACS bead positive selection kits (Milteny Biotec). 5x10^6 bone marrow cells were transplanted into donors. Bl6 mice received additionally 1x10^6 CD4/CD8 T cells and Balb/c mice received varying doses of 0,5, 1,0 and 5,0x10^6 CD4/CD8 T cells. Cells were resuspended in 300μl PBS in total and were injected retroorbitally.

To verify engraftment of bone marrow into donor animals, blood from mice was obtained by check bleeding und analyzed by flow cytometric analysis for MHC I marker specific for either Balb/c (H2k-d) or Bl6 (H2k-b).

3.3.5.2. Scoring

To examine and evaluate GvHD development in donor mice, animals were examined and scored daily for the following criteria: Bl6 weight loss 5-10% (5P), Bl6 weight loss 10-19% (10P), Bl6 weight loss >20% (20P), Balb/c weight loss 10-20% (5P), Balb/c weight loss 20-29% (10P), Balb/c weight loss >30% (20P), pale skin (5P), small hunchback (5P), big or for longer duration hunchback (10P), signs of infection (20P), beginning skin GvHD (5P), growing skin GvHD (10P), inflamed skin GvHD (20P), beginning diarrhea (5P), diarrhea up to 3 days (10P), diarrhea longer then 3 days (20P).

3.3.5.3. Disease induction analysis

To better compare differences between genotypes during GvHD induction in Bl6 mice, GvHD was induced as described before (3.3.6.1.) and mice were analyzed at day 8 after induction. Therefore, cell subsets in the lamina propria and intraepithelial compartment of colon and ileum were analyzed.

For isolation of cells from the intestinal intraepithelial compartment, colon and ileum were washed with cold PBS and cut into 2 cm pieces. Longitudinally opened intestines were incubated with HBSS solution containing 2mM EDTA, 10mM HEPES, 10% FCS (Capricorn), 1 % L-Glutamine and 1mM DTT (Sigma-Aldrich). After incubation on a shaker (225 rpm) at 37°C for 15 min, supernatants were filtered through a 100µm strainer. After another incubation step, supernatants were filtered, pooled and placed on ice (45 min). Cells in suspension were purified on a 40/80% Percoll gradient (Biochrom) and FACS analyzed for CD3, CD4, CD8, γδ TCR, IL-17, IFNγ and MHC-I H2kb and H2kd to distinguish between donor and recipient cells.

For the assessment of cell subsets in the lamina propria, colon and ileum were prepared as just described, but this time remaining tissue pieces after incubation with DTT solution were used and further digested for 45min in $PBS^{+Ca/+Mg}$ supplemented with FCS (10%), Collagenase II (200 U/ml; Worthington), and DNase I (0.05 mg/ml; Roche) on a shaker at 37°C. Cells in suspension were filtered through a 100µm strainer and purified on a 40/80% Percoll gradient (Biochrom). Cells in the lamina propria were again FACS

analyzed for T cell markers (CD3, CD4, CD8, γδ TCR, IL-17, IFNγ), and as well for MHC-I H2kb and H2kd to distinguish between donor and recipient cells.

3.4. Statistical analysis

GraphPad Prism V software was used for generating Kaplan-Meier curves and performing statistical analysis (using Mantle-Cox test) to compare the survival of mice. For further statistical analysis, p values were determined by applying two-tailed t-test for independent samples. All values are expressed as the mean ± SEM and $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.0005$ (***).

4. Results

4.1. Inflammatory cell death of *Xiap* $^{-/-}$ BMDCs triggers exaggerated IL-17 production in T cells

4.1.1. Experimental setup to elucidate inflammatory cell death on T cell polarization

In a recent publication, Yabal and colleagues (Yabal et al. 2014) showed that dendritic cells (DCs) deficient for XIAP are prone to increased cell death and showed a hyperinflammatory phenotype with increased IL-1β production after TLR treament. To investigate consequences of inflammatory cell death of stimulated dendritic cells on their surroundings, a coculture system with dendritic cells and T cells was developed (Fig.4.1).

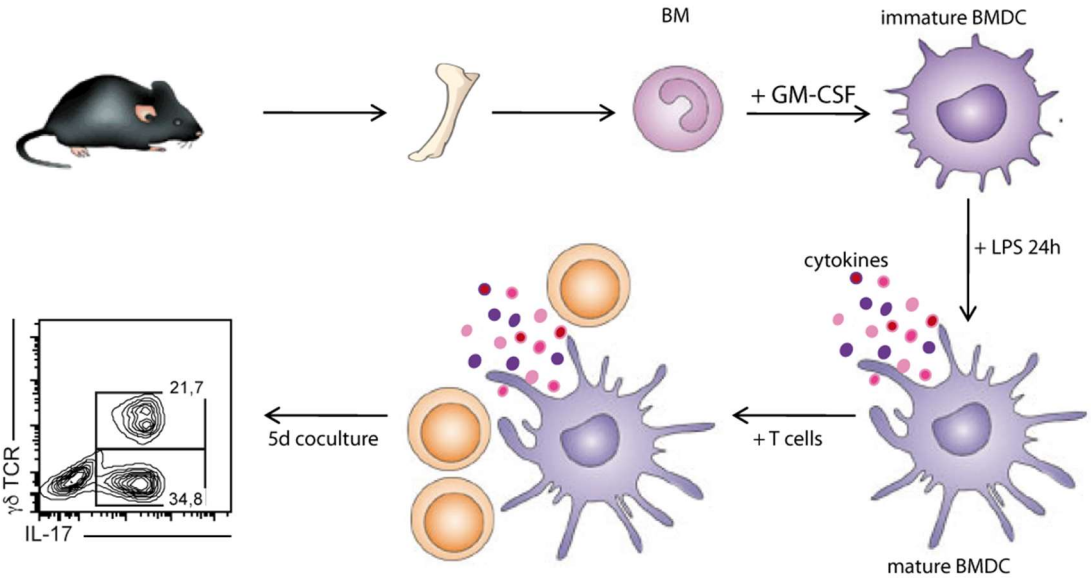


Figure 4. 1 *Ex vivo* experimental coculture setup.
Illustration of the experimental setup for the differentiation of BMDCs from bone marrow followed by stimulation of BMDCs with TLR ligands. Purification of T lymphocytes from spleen and lymphnodes and addition to stimulated BMDCs after 24h followed by flow cytometric analysis of T lymphocytes after 5 days of coculture.

Therefore, bone marrow (BM) from WT and XIAP deficient mice was differentiated with GM-CSF for seven days. Dendritic cells were then stimulated with various TLR ligands (LPS for TLR4, pI:C for TLR3 and CpG for TLR9) for 24 hours. After 24 hours T cells from WT mice were isolated and added to the stimulated DCs and were subsequently cocultured for the following 5 days. After coculture, T lymphocytes were subject to flow cytometric analysis.

4.1.2. GM-CSF differentiated cells are a heterogeneous group of dendritic cells and macrophages

To enable a better characterization of the dendritic cells used in the following experiments, differentiated BMDCs were subject to analysis. After seven days of bone marrow cell differentiation, surface markers of dendritic cells were analyzed for CD11b and CD11c (Fig.4.1A), which are known to be expressed on the surface of different dendritic cell subsets (Merad et al. 2013). CD11b/CD11c analysis revealed the existence of two subsets with the main population being $CD11b^{+}/CD11c^{+}$ and a minor subset ($CD11b^{int}/CD11c^{+}$) that increased after stimulation (Fig.4.2.A). This mirrors the populations described by Helft et al., who states that GM-CSF differentiated dendritic cells are not a uniform population, but rather a mixture of dendritic cells and macrophages (Helft et al. 2015). Therefore, further flow cytometric analysis was done to verify the macrophage like phenotype of the $CD11b^{+}/CD11c^{+}$ subset and the dendritic like features of the $CD11b^{int}/CD11c^{+}$ cells. The $CD11b^{+}/CD11c^{+}$ subset showed a high expression of F4/80 and 7/4, characterizing these cells as macrophages, while the $CD11b^{int}/CD11c^{+}$ subset depicted dendritic cells with features of high MHC-II and reduced 7/4 expression (Fig.4.2.B and 4.2.C). Inflammatory monocytes were not included, as this subset would express Gr-1 while being negative for CD11c expression (Dunay et al. 2008). Although differentiated BM cells include for the better part macrophages, they will still be termed dendritic cells, according to the differentiation protocol.

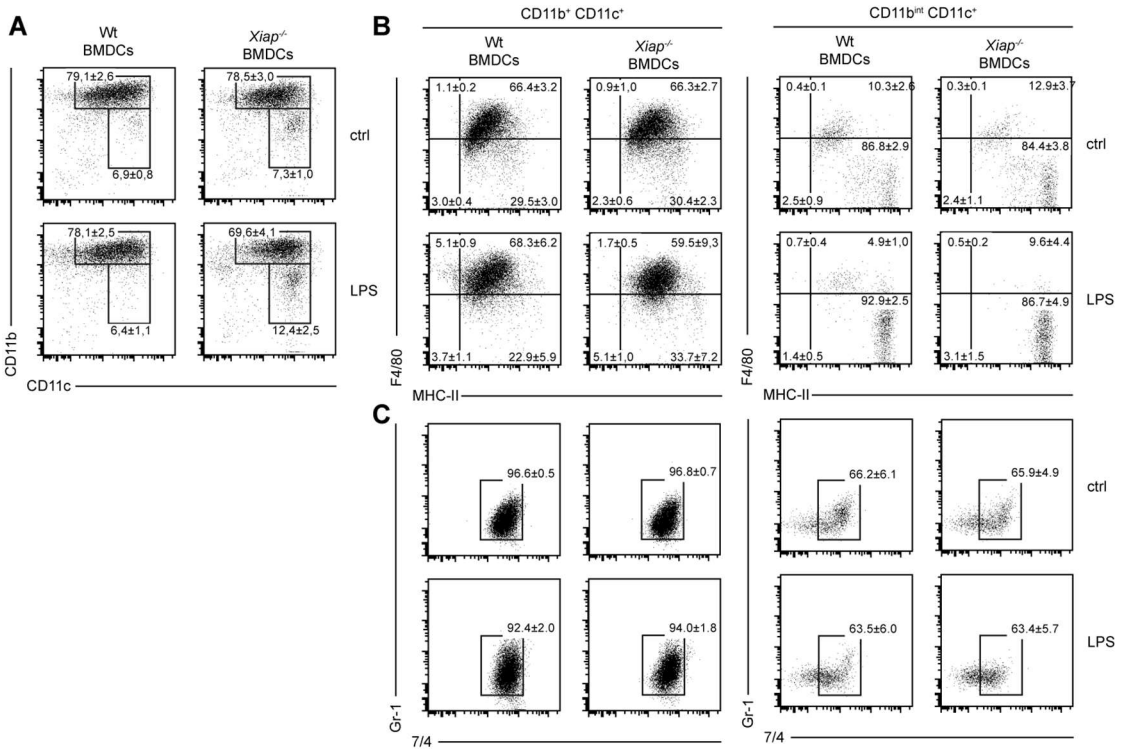


Figure 4. 2.: Flow cytometric analysis of dendritic cells.

Flow cytometric analysis of differentiated BMDCs shows different subsets of dendritic cells and macrophage like cells based on CD11b/CD11c expression (A), differences in F4/80 and MHC-II (B), as well as Gr-1 and 7/4 expression (C).

4.1.3.TLR triggered loss of viability in XIAP deficient BMDCs

First, bone marrow progenitors of WT mice were differentiated and compared to BMDCs lacking XIAP or expressing a RING-deleted version of XIAP termed *Xiap*$^{\Delta Ring/\Delta Ring}$. Viability of DCs was assessed after treatment with different toll-like receptor (TLR) stimuli (LPS for TLR4, pI:C for TLR3 and CpG for TLR9), resembling microbial loads after infection. 24 hours after treatment, survival of BMDCs was determined by intracellular ATP content. *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs both showed a significant decrease in viability in comparison to WT BMDCs (Fig.4.3.A). Moreover, we detected increased amounts of LDH release from *Xiap*$^{-/-}$ BMDCs (Fig.4.3.B), which correlates with lytic and therefore necrotic cell death (Chan, Moriwaki, and De Rosa 2013). Additionally, cell death of LPS treated *Xiap*$^{-/-}$ BMDCs could be clearly distinguished from

apoptotic death by propidium iodid (PI) stainings, which can only stain cells with ruptured membranes (Fig.4.3.C) (Krysko et al. 2008).

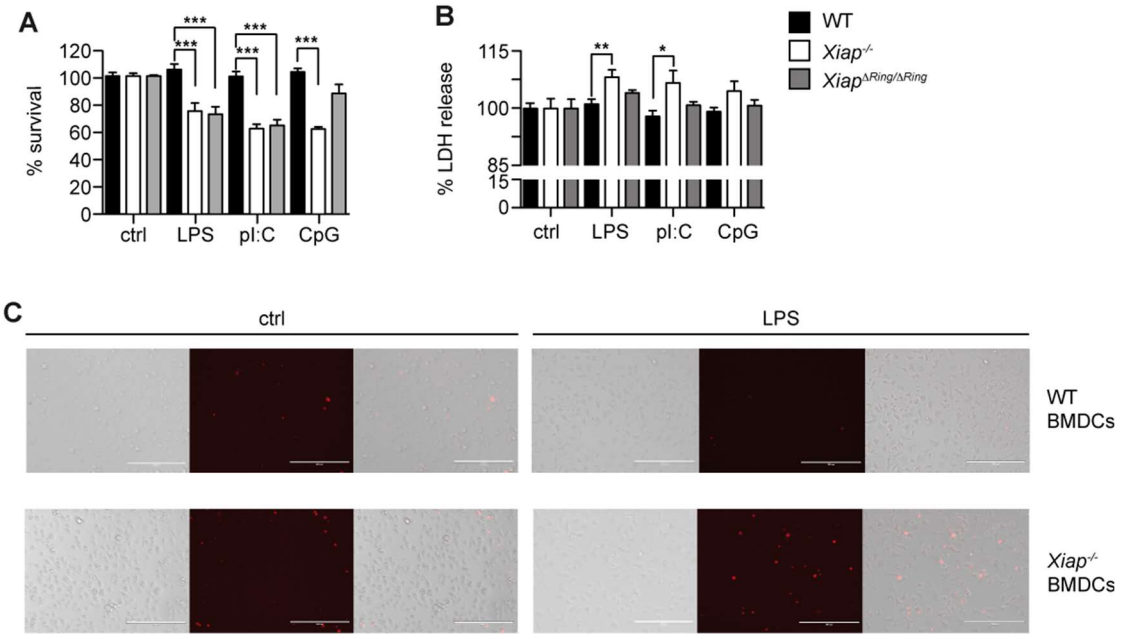


Figure 4. 3.: Detection and distinction of cell death after TLR treatment.
Statistical analysis was done by unpaired t-test and asterisks denote significant differences (*p < 0,05, **p < 0,005, ***p < 0,0005; p values valid for all subsequent figures). Measurement of cell death by decrease in ATP content (A), LDH release (B) and PI staining (C) in WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs after 24h of treatment with LPS (2ng), pI:C (10mg/ml) or CpG(10nM). PI staining was visualized under the transmitted light microscope (left pictures) with Texas Red fluorescence detection (middle pictures) and displayed as overlaid pictures (right).

4.1.4. Elevated amounts of proinflammatory cytokines in *Xiap* $^{-/-}$ BMDCs after TLR stimulation

In coherence with increased cell death of *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs, we measured proinflammatory cytokines after 24 hours. Secretion of IL-6 and TNF was comparable between WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs but there was an increased IL-23 secretion visible in XIAP deficient cells (Fig.4.4.A). However, most striking was the difference of IL-1β, which was absent in WT

BMDCs in contrast to *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs, independent of the given stimulus (Fig.4.4.A). As the death rate as well as the amounts of secreted cytokines were comparable between *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs we conclude that the phenotype is dependent on the E3 ligase activity of XIAP. To examine whether increased cytokine production was regulated on the transcriptional level, mRNA amounts were detected and showed a similar upregulation of inflammatory cytokines after four hours as well as 24 hours independent of the genetic background of the dendritic cells (Fig.4.4.B). Therefore, it can be deduced, that XIAP does not play a regulating role in the transcriptional control of these cytokines.

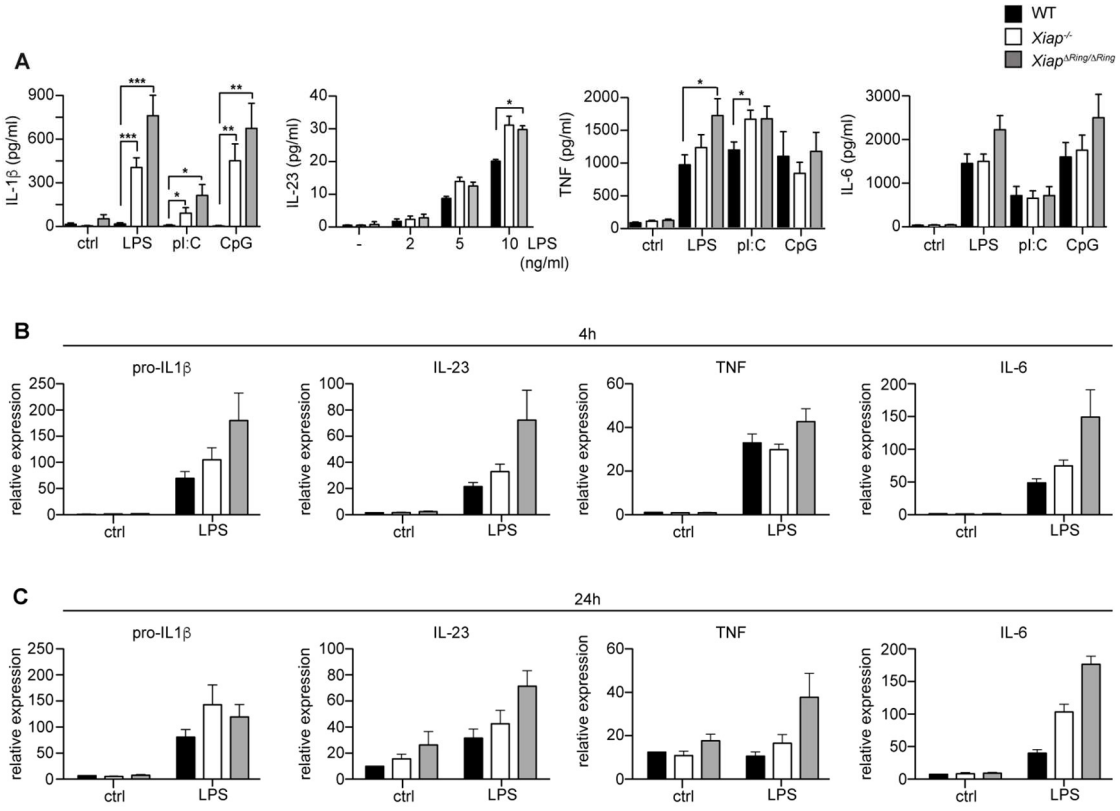


Figure 4. 4.: Cytokine induction and production of LPS treated BMDCs.

(A) Detection of secreted cytokines by LPS (2ng), pI:C (10mg/ml) or CpG(10nM) stimulated BMDCs after 24h. (B) Determination of the transcriptional induction by the same cytokines after 4h and 24h by relative mRNA expression.

4.1.5. Successful establishment of a BMDC – T lymphocyte coculture system

With the striking upregulation of inflammatory cytokines, after cell death in XIAP deficient cells, the aim was to determine whether inflammatory cell death could have an impact on the surrounding tissue including T lymphocytes and their polarization. Thus, T lymphocytes from the spleen and lymph nodes of a WT mouse were isolated and purified by a MACS bead negative selection kit. The kit used was described to enrich for $CD4^+$ T lymphocytes, but due to a missing antibody against $\gamma\delta$ T cells, $\gamma\delta$ T cells were also separated in addition to $CD4^+$ T lymphocytes (Fig.4.5.A). Purification yielded around 92% $CD4^+$ cells and 3-4% $\gamma\delta$ T lymphocytes. These lymphocytes were added to 24 hours TLR stimulated WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs and were subject to flow cytometric analysis after five days of coculture. The gating strategy in Figure 4.5.B shows that even after coculture a clear $CD4^+$ and $CD4^-$ subset remained, whereupon the $CD4^-$ subset clearly increased in size (from 7-8% up to 30%). Although we added no T cell receptor stimulating ligands to our coculture we saw only 10-20% of dead T lymphocytes ($Amin^+$) after coculture (Fig.4.5.C). Nevertheless, even without the addition of antigens, interaction of T lymphocytes with dendritic cells was essential for T cell survival. Culturing purified T cells with only the supernatant from stimulated dendritic cells, was not sufficient to keep the lymphocytes alive (Fig.4.5.D).

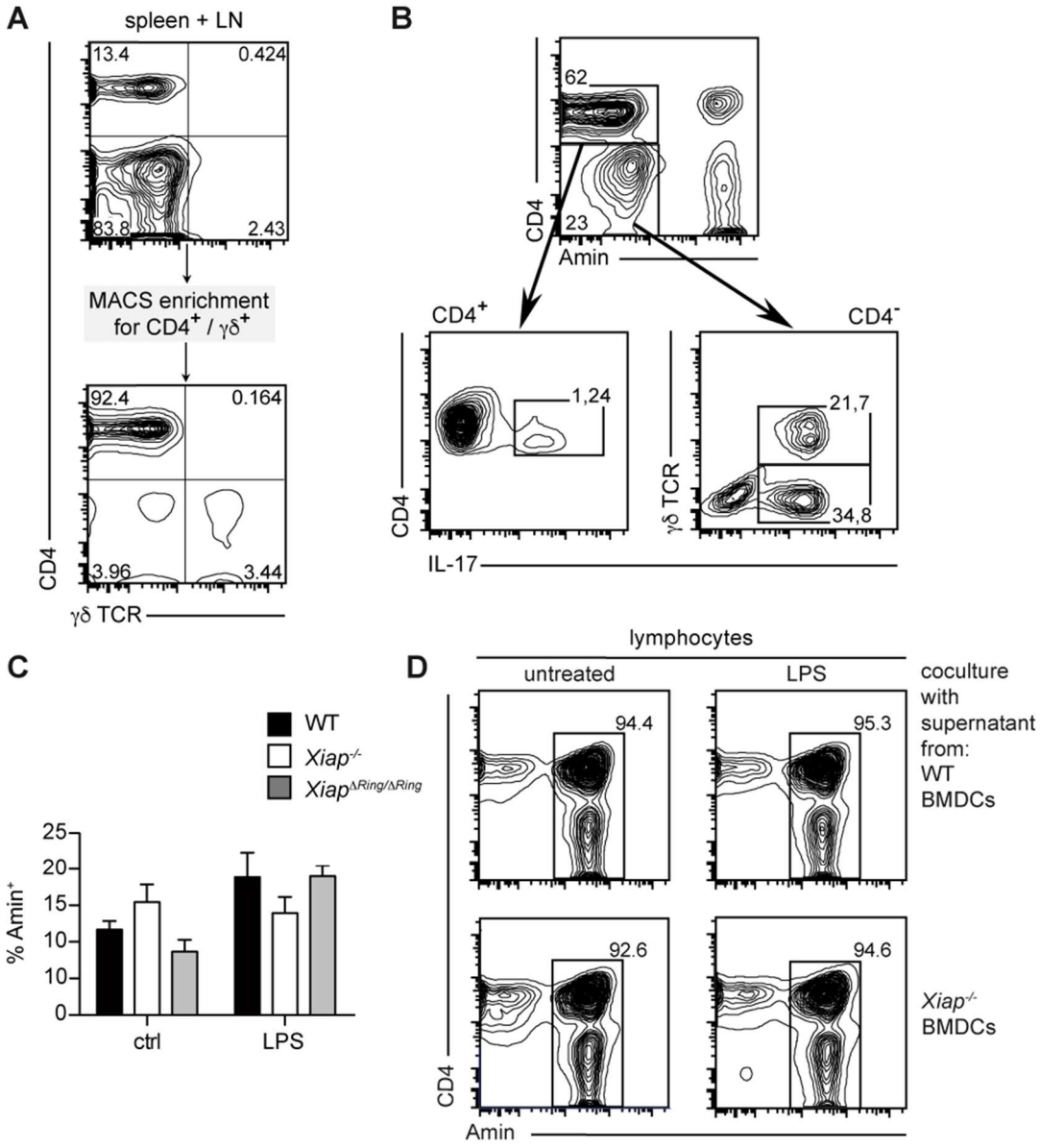


Figure 4. 5.: T lymphocyte characterization before and after coculture.

(A) Flow cytometric analysis shows CD4 and γδ T lymphocytes before and after purification with MACS beads. (B) Gating strategy for flow cytometric analysis after coculture. (C) Number of dead lymphocytes ($Amin^+$) after coculture. (D) Flow cytometric analysis T lymphocytes of one exemplary coculture with only the supernatant of untreated (ctrl) and LPS treated WT and *Xiap*$^{-/-}$ BMDCs.

4.1.6. Inflammatory cell death of *Xiap*$^{-/-}$ BMDCs triggers minor but significant IL–17 production in CD4$^+$ lymphocytes

According to the gating strategy depicted in Figure 4.5.B, CD4$^+$ and CD4$^-$ T lymphocytes could be distinguished. Analyzing intracellular cytokine expression revealed, that CD4$^+$ lymphocytes which were in coculture with TLR

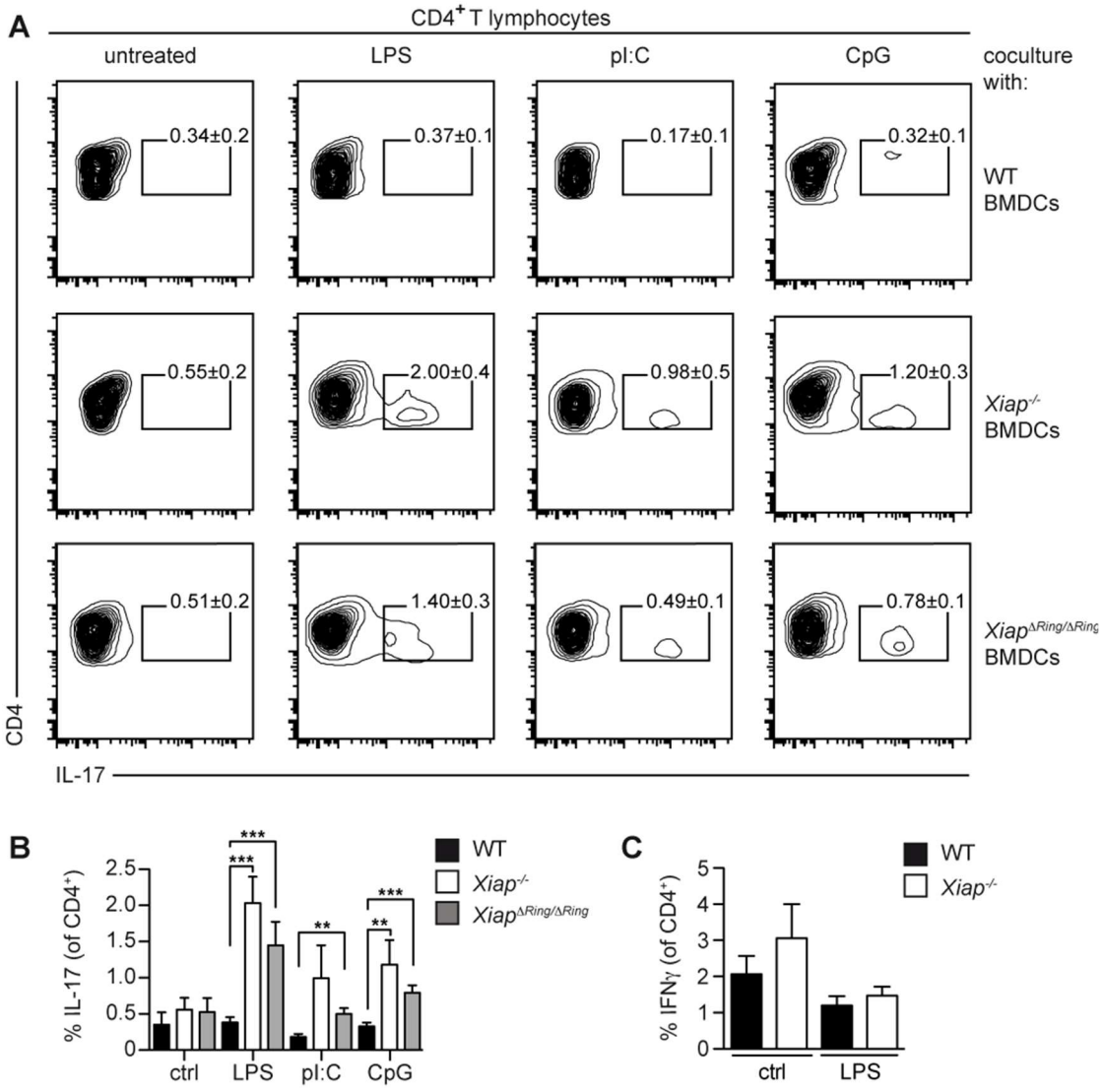


Figure 4. 6.: IL-17 induction in CD4+ T lymphocytes after coculture.

(A) Flow cytometric analysis of CD4$^+$ T lymphocytes after coculture with untreated, LPS (2ng), pI:C (10mg/ml) or CpG(10nM) treated WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs (B) Percentage of IL-17 induction in WT CD4$^+$ T lymphocytes after 5 days of coculture with treated (as described in A) WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs (C) Percentage of IFNγ expression in WT CD4$^+$ T lymphocytes after 5 days of coculture with treated (as described in A) WT and *Xiap*$^{-/-}$ BMDCs.

ligand treated *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs showed an increased expression of IL-17 from 0,5% up to 2% (Fig.4.6.A, 4.6.B). This was not detectable in untreated controls and T cells from coculture with WT BMDCs. Moreover, coculture did not influence IFNγ expression (Fig.4.6.C), a prototypic CD4$^+$ Th1 cytokine (Nembrini et al. 2006).

4.1.7. Inflammatory cell death of *Xiap*$^{-/-}$ BMDCs triggers excessive IL-17 production in CD4$^-$ lymphocytes

In addition to the CD4$^+$ T cell population, the CD4$^-$ counterpart was also analyzed with regard to intracellular cytokines. As purification of T cells was independent of γδ TCR expression, γδ$^+$ T cells as well as γδ$^-$ T cells were part of the CD4$^-$ subset and showed a similar phenotype as seen with CD4$^+$ cells, only much more pronounced (Figure 4.7.A,). After coculture with treated *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs, γδ$^+$ T cells and γδ$^-$ T cells showed an increased expression of IL-17 of up to 35%, which was not detectable in controls and with cocultured WT BMDCs (Figure 4.7.B, 4.7.C). Identical to CD4$^+$ cells, no IFNγ expression was triggered neither in γδ$^+$ nor in γδ$^-$ subsets after coculture with WT or *Xiap*$^{-/-}$BMDCs (Figure 4.7.D). To verify that the intracellular presence of IL-17 also translated into an actively secreted inflammatory cytokine, IL-17 was measured in the supernatant of the coculture. The intracellular phenotype was confirmed, showing concentrations of up to approximately 4ng/ml IL-17 in the supernatant of cocultures with *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs (Figure 4.7.E).

In summary, it was show that presence of a microbial encounter in case of XIAP deficiency or loss of XIAP´s E3 ligase activity leads to inflammatory cell death which induces IL-17 production, but not IFNγ, in surrounding T lymphocytes.

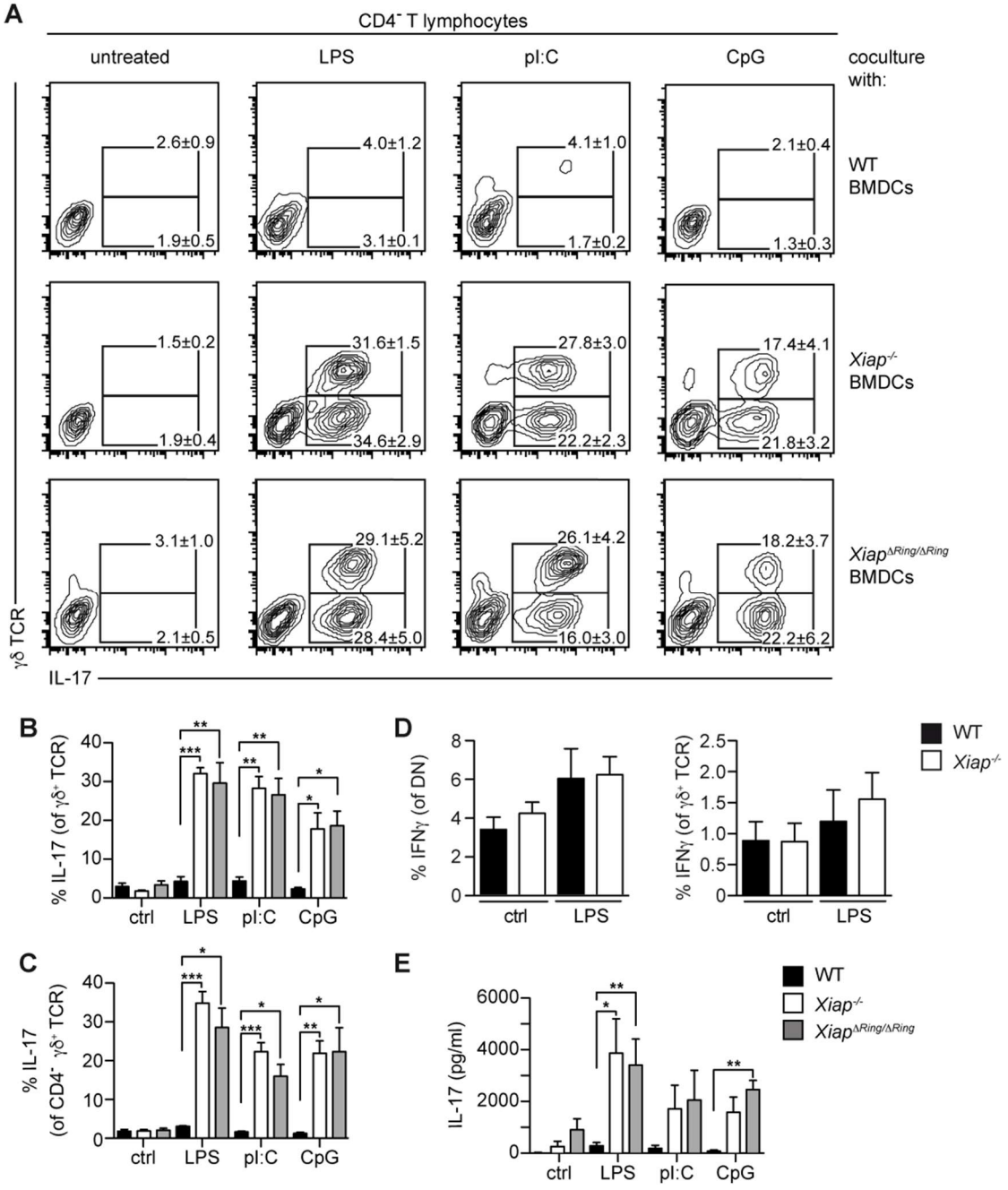


Figure 4. 7.: IL-17 induction in CD4⁻ T lymphocytes after coculture.

(A) Flow cytometric analysis of CD4⁻ T lymphocytes after coculture with untreated, LPS (2ng), pI:C (10mg/ml) or CpG(10nM) treated WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs (B) (C) Percentage of IL-17 induction in $\gamma\delta^+$ and $\gamma\delta^-$ T lymphocytes after 5 days of coculture with treated (as described in A) WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs (D) Percentage of IFNγ expression in $\gamma\delta^+$ and $\gamma\delta^-$ T lymphocytes after 5 days of coculture with LPS (2ng/ml) treated WT and *Xiap*$^{-/-}$ BMDCs. (E) IL-17 measured from supernatant after coculture with ELISA.

4.1.8. Irradiation induced apoptotic death is not inflammatory

To compare the phenotype of inflammatory death observed after TLR ligation in XIAP deficient cells with the effects of apoptosis, further experiments were conducted. WT and *Xiap*$^{-/-}$ BMDCs were subjected to UV irradiation of increasing doses, as UV irradiation is known to be a classical inducer of the intrinsic and extrinsic apoptotic pathway (C.-H. Lee et al. 2013; Scoltock and Cidlowski 2004). 24 hours after irradiation, increasing rates of death were detectable, correlating with the intensity of irradiation, which were comparable between WT and *Xiap*$^{-/-}$ BMDCrs (Fig.4.8.A). Apart from a basal, stimulation independent, TNF production, no notable IL-6 or IL-1β could be detected in the supernatant of WT and *Xiap*$^{-/-}$ BMDCs after irradiation (Fig.4.8.B).

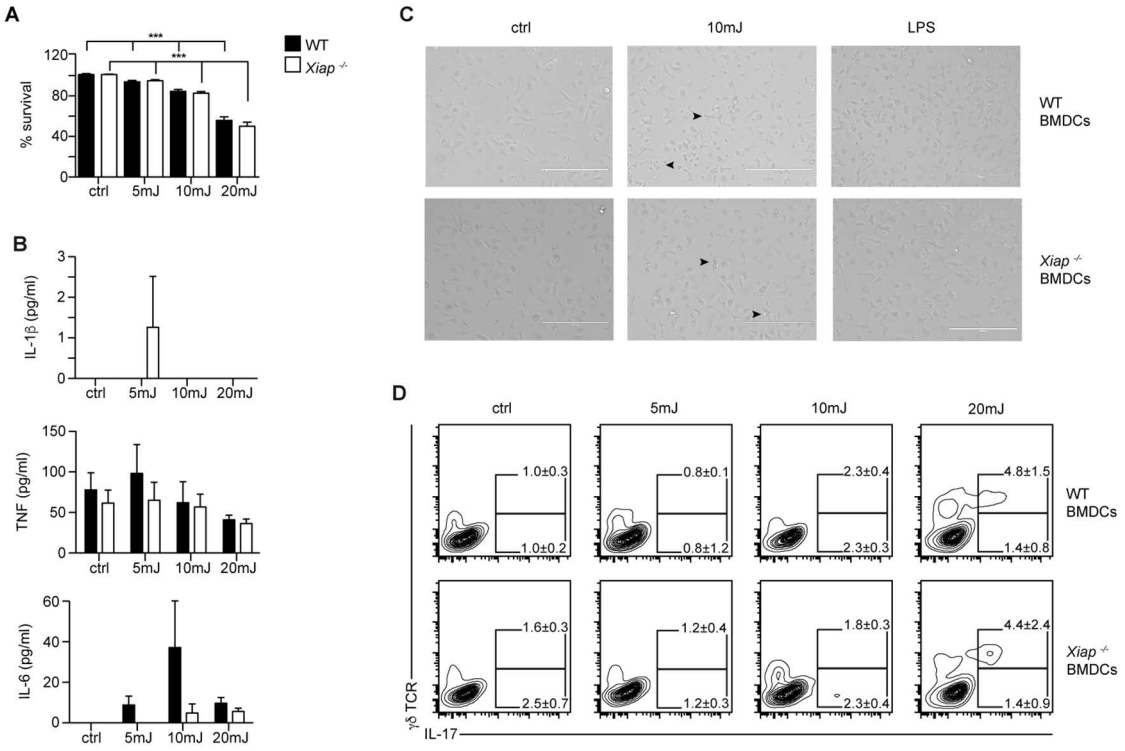


Figure 4. 8.: UV induced cell death is not inflammatory and has no effect on T lymphocytes.

(A) Survival of WT and *Xiap*$^{-/-}$ BMDCs 24h after UV irradiation of increasing dosages (5mJ/cm^2, 10mJ/cm^2, 20mJ/cm^2) (B) Secreted IL-1β, TNF and IL-6 of WT and *Xiap*$^{-/-}$ BMDCs after 24h of UV irradiation (C) Transmitted light microscopy pictures taken after 8h of WT and *Xiap*$^{-/-}$ BMDCs with either 10mJ/cm^2 UV irradiation or LPS (2ng(ml) treatment. Black arrows indicate apoptotic bodies. (D) Flow cytometric analysis of CD4$^-$ T lymphocytes after coculture with untreated or UV irradiated WT and *Xiap*$^{-/-}$ BMDCs.

In addition, WT and *Xiap*$^{-/-}$ BMDCs were compared under the transmitted light microscope, where apoptotic bodies after UV irradiation, but not after LPS treatment were visible. Consequently, a coculture of irradiated BMDCs with T lymphocytes was set up, but no IL-17 induction was detectable in T lymphocytes, whether cocultured with irradiated WT or *Xiap*$^{-/-}$ BMDCS (Fig..4.8.D).

4.2. Diverse innate T cell subsets react upon inflammatory cell death

4.2.1. Characterization of IL-17 producing cells reveals dominance of innate like T lymphocytes

To find out which T lymphocyte populations exactly showed IL-17 induction in the coculture system, in depth flow cytometric analysis was performed. With only 2% IL-17 positivity in CD4$^+$ T lymphocytes (Fig.4.6) in comparison to up to 50% IL-17 positivity in CD4$^-$ T lymphocytes (Fig.4.7), the focus was to understand which non-CD4 cells were responsible for the strong IL-17 production and additionally showed proliferative capabilities (Fig.4.5.B, 4.5.E). Initial flow cytometric analysis showed that the CD4$^-$ subset of IL-17 producing T lymphocytes consisted of two groups. Despite enrichment for $\gamma\delta^+$ T lymphocytes during MACS bead purification, a bigger sized $\gamma\delta^-$ subset with similar capacities to induce IL-17 was observed (Fig.4.7). Further flow cytometric analysis confirmed that this subset consisted of T lymphocytes with expression of CD3 and TCRβ. CD8$^+$ T lymphocytes and NK cells could be excluded (Fig.4.9.A), even though they are two known subsets being capable of IL-17 production (Srenathan, Steel, and Taams 2016; Passos et al. 2010). Although iNKT cells usually display NK1.1 as a surface marker (Makino et al. 1995), more recent findings describe a NK1.1$^-$, but IL-17 producing subset of iNKT lymphocytes, termed iNKT17 (Michel et al. 2007). To identify possible contaminations of the T lymphocytes with iNKT cells, a stain for the invariant T cell receptor was established and enhanced with intracellular stainings for

the IL-17 specific transcription factor RoRγt (Ivanov et al. 2006; Michel et al. 2008) and the iNKT staining for RoRγt and (innate like T cell) specific

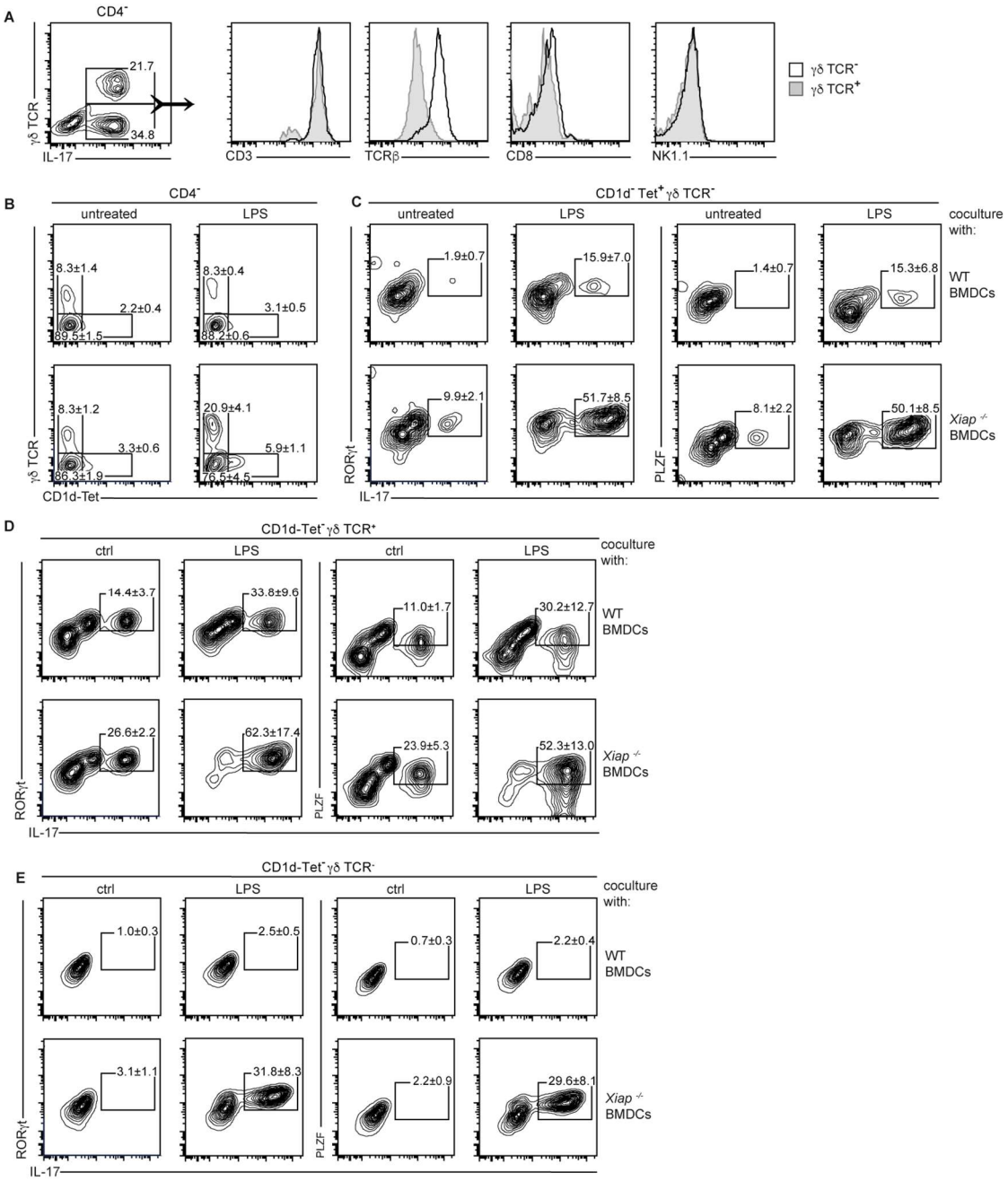


Figure 4. 9.: IL-17 producing T cells belong to innate like T lymphocytes.

(A) Flow cytometric analysis of CD4⁻ IL17⁺ T lymphocytes subsets after coculture with LPS (2ng/ml) treated *Xiap*$^{-/-}$ BMDCs distinguishing between γδ^{+} and γδ^{-} T lymphocytes and their expression of CD3, TCRβ, CD8 and NK1.1 (B) Flow cytometric analysis of CD4^{-} T lymphocytes to distinguish γδ^{+} T cells and iNKT17 cell after coculture with LPS (2ng/ml) treated WT and *Xiap*$^{-/-}$ BMDCs (C) (D) (E) Intracellular PLZF of γδ^{-} iNKT^{+}(C), γδ^{+} iNKT^{-} (D) and γδ^{-} iNKT^{-} (E) found subsets in (B)

transcription factor PLZF (Constantinides and Bendelac 2013). Flow cytometric analysis confirmed the presence of iNKT cells in the coculture, which also showed, like γδ T cells, an expansion after coculture with stimulated *Xiap*$^{-/-}$ BMDCs (Fig.4.9.B). Further stainings for the two mentioned transcription factors, helped to classify the iNKT subset (Engel et al. 2016) and to verify the presence of iNKT17 lymphocytes with the expression of PLZF and RoRγt in correlation with increasing IL-17 following coculture with stimulated *Xiap*$^{-/-}$ BMDCs (Fig.4.9.C). Interestingly, not only iNKT17 cells were positive for the transcription factors, but also γδ^{+} showed high RoRγt and intermediate PLZF expression (Fig.4.9.D) and the remaining γδ^{-}iNKT^{-} lymphocytes were likewise double positive (Fig.4.9.E). Given that the iNKT17 lymphocytes were only a small fraction of the IL-17 producing subset (only up to 6%), and due to the extensive flow cytometric analysis it was concluded that the remaining γδ^{-} iNKT^{-} lymphocytes were indeed peripheral double negative (DN) lymphocytes. Since the iNKT17 presence was so small, it will not be differentiated from the DN T lymphocytes for the following *in vitro* experiments.

4.2.2. Peripheral DN T cells are a distinct IL-17 producing subset

To evaluate IL-17 induction during the proliferation of T lymphocytes, CFSE staining was performed. With the membrane intercalating CFSE dye, cell division tracking was possible, revealing that IL-17 positivity is already strongly induced in CD4^{-} T lymphocytes at day 3 and continues to increase up to day 5 (Fig.4.10.A). Examining all viable T lymphocytes with the CFSE stain shows a small but detectable subset of CD4^{+} and a larger group of CD4^{-} cells, which divided during the course of the five days even in the untreated controls (Fig.4.10.B). Specifically, the CD4^{-} subset cocultured with *Xiap*$^{-/-}$ BMDCs after LPS treatment showed an increased number of cell divisions. Addition of antiCD3, to trigger the TCR receptor, resulted in a strong proliferation of the CD4^{+} subset, but did not have an impact on the CD4^{-} subpopulation, independent of additional LPS stimulation (Fig.4.5.E). Nevertheless, with the

CFSE stain it cannot be truly distinguished whether the emerging and growing CD4⁻ subset (specifically the DN T cells) is simply a strongly growing group of

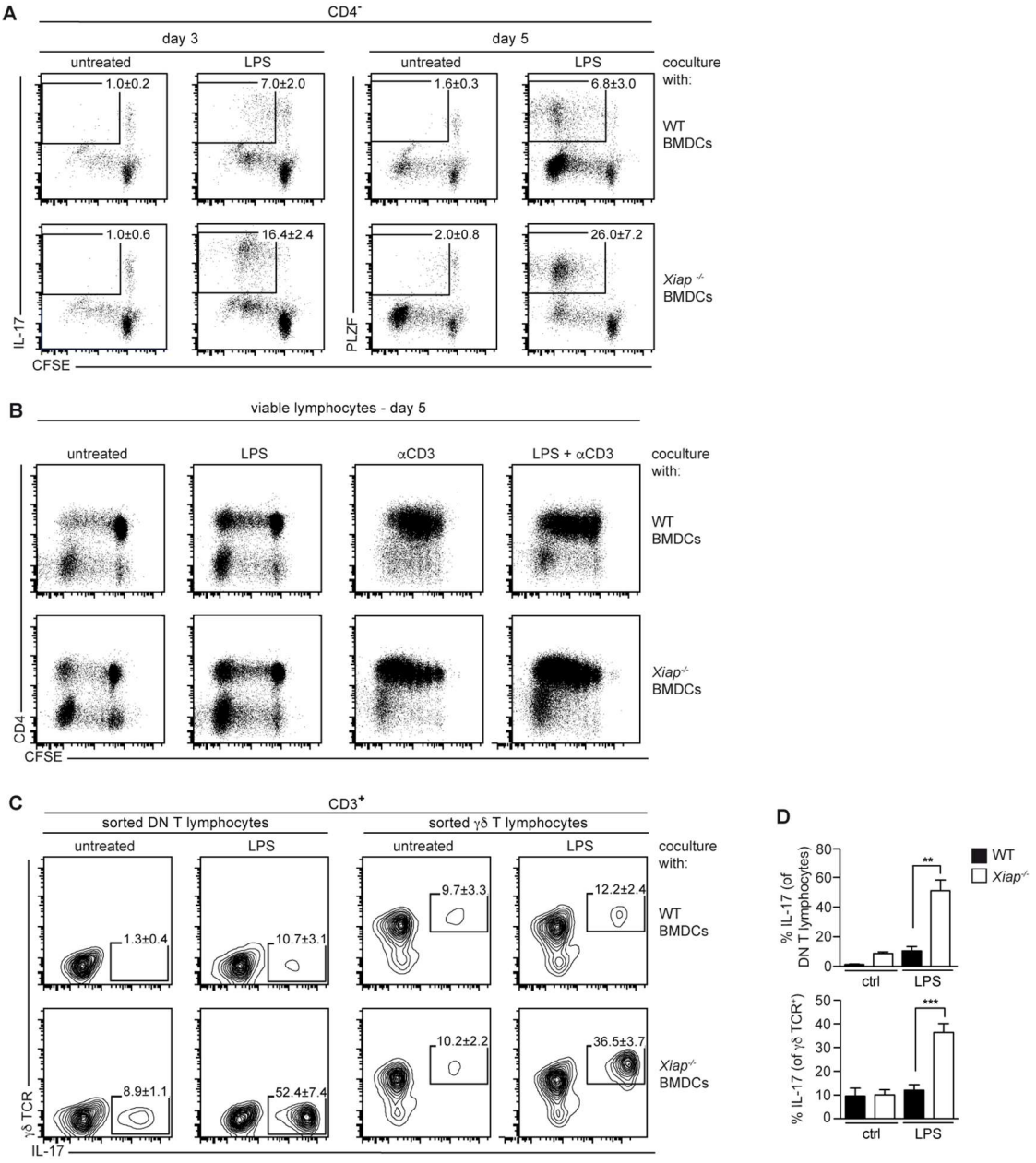


Figure 4. 10.: DN IL-17 producing lymphocytes do not originate from CD4 T cells.

(A) Flow cytometric analysis of WT CD4⁻ T cells for CFSE cell proliferation at day 3 and day 5 in coculture with LPS (2ng/ml) treated WT and *Xiap*$^{-/-}$ BMDCs (B) CFSE staining of T lymphocytes after 5 days of coculture with untreated, LPS, antiCD3 and LPS+antiCD3 treated WT and *Xiap*$^{-/-}$ BMDCs. (C) (D) Flow cytometric analysis and percentage of IL-17 induction of WT DN (γδ⁻CD4⁻CD8⁻CD3⁺) and γδ⁺ T cells after purification via fluorescence–activated cell sorting and 5 days of coculture with LPS (2ng/ml) treated WT and *Xiap*$^{-/-}$ BMDCs.

γδ and DN T lymphocytes, or the increasing DN subpopulation is a result of CD4 receptor downregulation. This process has been described before after chronic stimulation (Grishkan et al. 2013) and displays one theory how DN T lymphocytes originate. To address the question of DN T cell origin in our coculture, fluorescence – activated sorting was performed and resulted in highly pure cell populations of either $\gamma\delta^+$ or DN ($CD3^+$, $\gamma\delta^-$, $CD4^-$, $CD8^-$) T lymphocytes. The purified T cells were then added to the stimulated BMDCs and analyzed after 5 days of coculture. Evaluation revealed that purified DN lymphocytes and γδ T cells expanded and produced even higher IL–17 amounts, independently of $CD4^+$ lymphocytes (Fig.4.10.C) and thus DN T cells do not originate from $CD4^+$ T lymphocytes.

4.2.3.XIAP in T lymphocytes is not involved in polarization

So far, only T lymphocytes purified from WT mice were used. This does not reflect the situation in an XLP-2 patient, were not only the dendritic cells, but all cells are deficient for XIAP. To test the role of endogenous XIAP in T lymphocyte polarization, naïve T cells from WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ mice were purified and differentiated into Th17, Th1 and Treg T lymphocytes, using cytokines and blocking antibodies. Independent of the genetic background, the naïve T lymphocytes differentiated into the specific effector subset (Fig.4.11.A). Moreover, the behavior of XIAP deficient T lymphocytes in a coculture setup with WT and *Xiap*$^{-/-}$ BMDCs was examined and induction of IL-17 was analyzed. *Xiap*$^{-/-}$ T cells behaved identical to the WT T cells, showing comparable elevated levels of IL-17 in all three T cell subsets after coculture with *Xiap*$^{-/-}$ BMDCs (Fig.4.11.B) . Next, it was examined whether IL-17 induction was as well possible in an allogenic setting. Thus, T cells from WT Balb/c mice were purified as described before and were then scanned for IL-17 and IFNγ production after coculture. The quantity of IL-17^+ T cells was comparable between the syngenic setup with Bl6 T cells only and the allogenic setup of Bl6 BMDCs and Balb/c T cells (Fig.4.11.C). In contrast to the syngenic setup, there was an additional increase in IFNγ^+ $CD4^+$ T cells dependent on the coculture with treated *Xiap*$^{-/-}$ BMDCs (Fig.4.11.C). All in all, it can be

summarized that especially innate like T cells respond to inflammatory cell death in the absence of XIAP and initiate an inflammatory reaction, independent of their own genetic background.

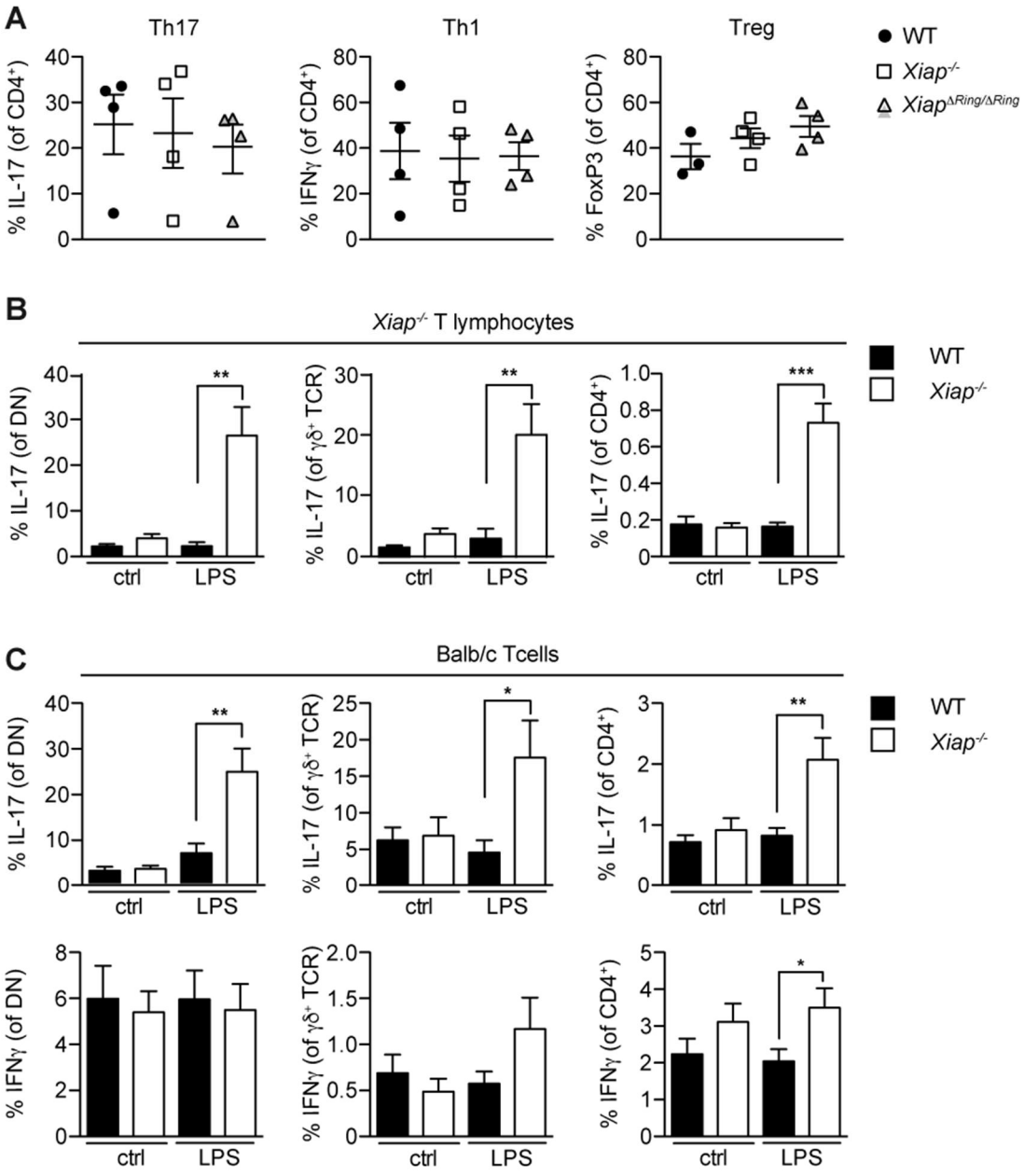


Figure 4. 11.: XIAP deficiency does not influence T cell differentiation.

(A) Flow cytometric analysis showing differentiation of Th17, Th1 and Treg cells via their respective intracellular signature cytokines (B) Percentage of IL-17 DN^+, $\gamma\delta^+$ and $CD4^+$ T cells with an XIAP deficient background after 5 days of coculture with LPS (2ng/ml) treated WT or *Xiap*$^{-/-}$ BMDCs (C) Percentage of IL-17/IFNγ DN^+, $\gamma\delta^+$ and $CD4^+$ T cells with a Balb/c genetic background after 5 days of coculture with LPS (2ng/ml) treated WT or *Xiap*$^{-/-}$ BMDCs.

4.3. TNF and Caspase dependent hyper-inflammatory IL-1β by *Xiap* $^{-/-}$ BMDCs activates T cells

4.3.1.IL-1β receptor signaling drives IL-17 induction in innate like T cells

To understand how inflammatory cell death can drive the induction of IL-17 in T lymphocytes further experiments were conducted. According to literature, polarizing cytokines are decisive for the differentiation status of a T lymphocyte. These can either be self-produced or come from antigen presenting cells (APCs) (Swain 1995). Several cytokines are known to drive Th17 polarization, with the combination of IL-6 and TGF-β being most important (Bettelli et al. 2006; Veldhoen et al. 2006). Moreover, IL-23 has been shown to support maturation and thereby late differentiation (McGeachy et al. 2009), whereas IL-1β facilitates expansion (Gulen et al. 2010) and early differentiation (Chung et al. 2009). On the other hand, innate like T cell subsets like γδ T lymphocytes seem to be able to induce IL-17 irrespective of IL-6, but rather depend on IL-1β and IL-23 (Sutton et al. 2009). As increased IL-1β and IL-23 are detectable after *Xiap*$^{-/-}$ BMDC TLR treatment, it was tested whether those cytokines were responsible for the IL-17 induction. Addition of Anakinra, an IL-1 receptor antagonist, with LPS did neither influence BMDC viability (Fig.4.12.A), nor did it have an autocrine effect on cytokine production (Fig.4.12.B), as the amount of viability and produced cytokines remained constant. In contrast to this, adding Anakinra in the coculture resulted in the complete loss of IL-17 induction in $CD4^-$ T lymphocytes (Fig.4.12.C, 4.12.D) as well as in $CD4^+$ T lymphocytes (Fig.4.12.E). This correlation of IL-1β signaling in T cells as a necessity for IL-17 induction was further affirmed by the fact, that T lymphocytes without a receptor for IL-1β were not able to respond to the death driven inflammation of *Xiap*$^{-/-}$ dendritic cell and did therefore not produce IL-17 (Fig.4.12.F). To prove that for the initiation

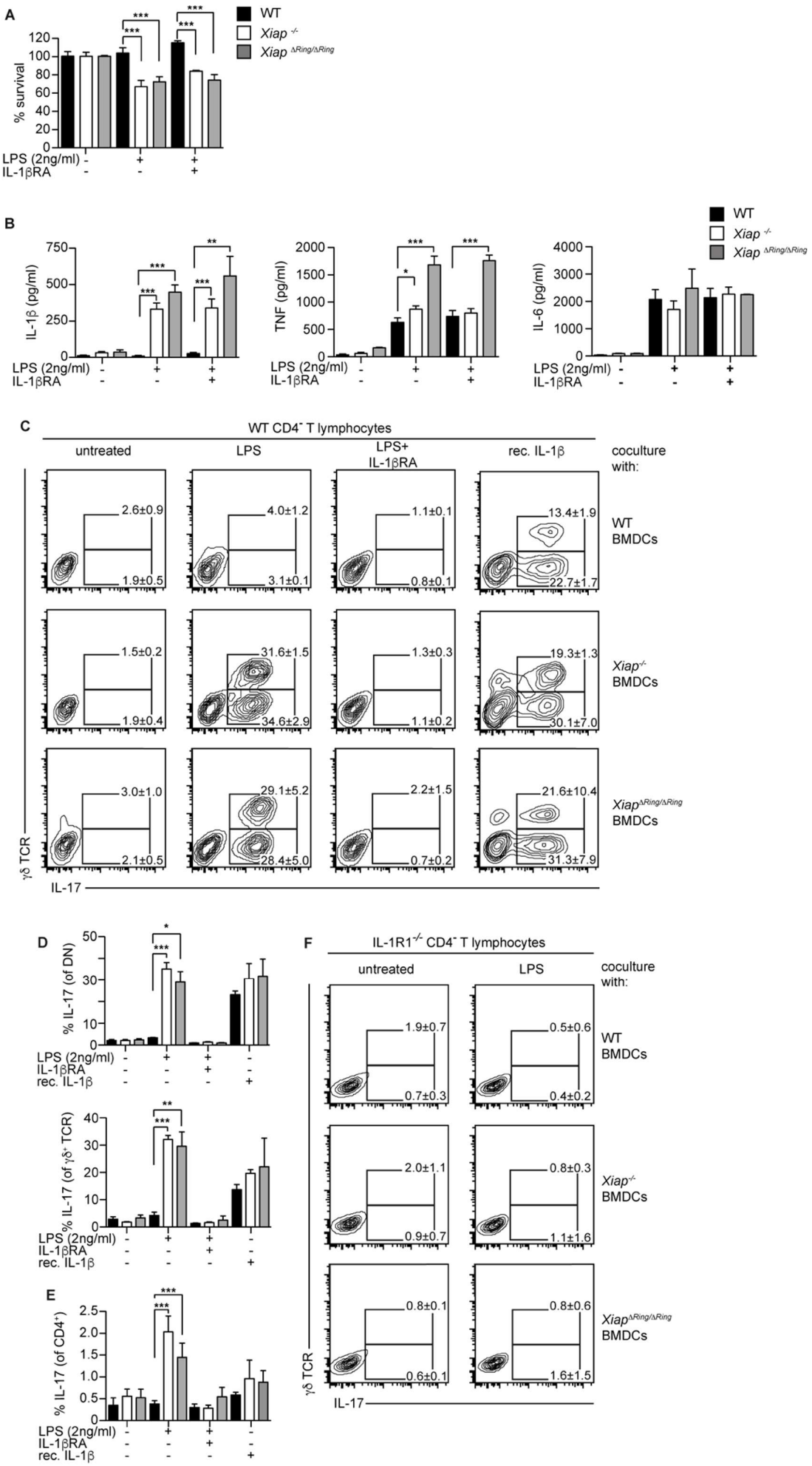

A
WT
Xiap -/-
Xiap ΔRing/ΔRing
% survival
LPS (2ng/ml)
IL-1βRA
B
IL-1β (pg/ml)
TNF (pg/ml)
IL-6 (pg/ml)
C
WT CD4- T lymphocytes
untreated
LPS
LPS+ IL-1βRA
rec. IL-1β
coculture with:
WT BMDCs
Xiap-/- BMDCs
XiapΔRing/ΔRing BMDCs
γδ TCR
IL-17
2.6±0.9
1.9±0.5
4.0±1.2
3.1±0.1
1.1±0.1
0.8±0.1
13.4±1.9
22.7±1.7
1.5±0.2
1.9±0.4
31.6±1.5
34.6±2.9
1.3±0.3
1.1±0.2
19.3±1.3
30.1±7.0
3.0±1.0
2.1±0.5
29.1±5.2
28.4±5.0
2.2±1.5
0.7±0.2
21.6±10.4
31.3±7.9
D
% IL-17 (of DN)
% IL-17 (of γδ+ TCR)
rec. IL-1β
E
% IL-17 (of CD4+)
F
IL-1R1-/- CD4- T lymphocytes
1.9±0.7
0.7±0.3
0.5±0.6
0.4±0.2
2.0±1.1
0.9±0.7
0.8±0.3
1.1±1.6
0.8±0.1
0.6±0.1
0.8±0.6
1.6±1.5

Figure 4. 12.: IL-1β signaling is the driving factor for IL-17 induction.

(A) Survival of WT, Xiap$^{-/-}$ and Xiap$^{\Delta Ring/\Delta Ring}$ BMDCs after 24h of LPS (2ng/ml) and additional IL-1β Receptor Antagonist (IL-1βRA) Anakinra (15mg/ml). (B) Cytokines of WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs after 24h of LPS (2ng/ml) and additional IL-1β Receptor Antagonist (IL-1βRA) Anakinra (15mg/ml). (C) Flow cytometric analysis of IL-17 in WT CD4$^-$ T cell subset after 5 days of coculture with LPS (2ng/ml), LPS + Anakinra (15mg/ml) and recombinant IL-1β (2ng/ml) treated WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs. (D) and (E) Percentage of intracellular IL-17 induction in WT CD4$^-$ and CD4$^+$ T cells of flow cytometric analysis depicted in 4.12.C, DN stands for DN T lymphocytes in all following figures. (F) Flow cytometric analysis of IL-1R1$^{-/-}$ CD4$^-$ T lymphocyte subset after 5 days of coculture with LPS (2ng/ml) treated WT, *Xiap*$^{-/-}$ and *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs.

of IL-17 in innate T lymphocyte subsets, IL-1β was the driving factor, recombinant IL-1β was added to the coculture system. After addition of recombinant IL-1β, a strong intracellular IL-17 signal (Fig.4.12.C, 4.12.D) in the innate T lymphocytes could be detected. On the contrary, recombinant IL-1β alone was not sufficient to induce IL-17 in CD4$^+$ T lymphocytes (Fig.4.12.E). It can be concluded, that inflammatory cell death which occurs after loss of XIAP results in elevated IL-1β which can directly affect T lymphocytes to induce IL-17. This IL1β signaling is necessary and sufficient for innate like T lymphocytes, while it is necessary but not sufficient for CD4$^+$ T cells.

4.3.2. Coculture with BMDMs mimics results from BMDC coculture

Flow cytometric analysis of differentiated BMDCs revealed a mixed population of cells with characteristics of dendritic cells but also macrophage features (Figure 4.2). Given that the differentiated BMDCs included macrophage like cells, a comparison between the behavior of BMDCs and bone marrow derived macrophages (BMDMs) made sense. Thus, BMDMs were differentiated

according to their protocol and were then treated like BMDCs. A striking difference between BMDMs and BMDCs was uncovered when treatment with 2ng/ml LPS did not result in detectable cell death in BMDMs and could also not be induced with an increased LPS concentration (Fig.4.13.A). Nevertheless, 24 hours LPS treatment yielded the production of inflammatory cytokines in similar quantities as measured in BMDC supernatant, with the same phenotype of strong IL-1β production, seen only by *Xiap*$^{-/-}$ BMDCs and not WT BMDCs (Fig.4.13.B). Again, Anakinra treatment did neither influence BMDC viability, nor cytokine production. When BMDMs were used for coculture setup, the IL-1β dependent phenotype was verified. Coculture of *Xiap*$^{-/-}$ BMDCs resulted in a similar, although not so strong, induction of IL-17 production in γδ^{+} and γδ^{-} (DN) T lymphocytes (Fig.4.13.C).

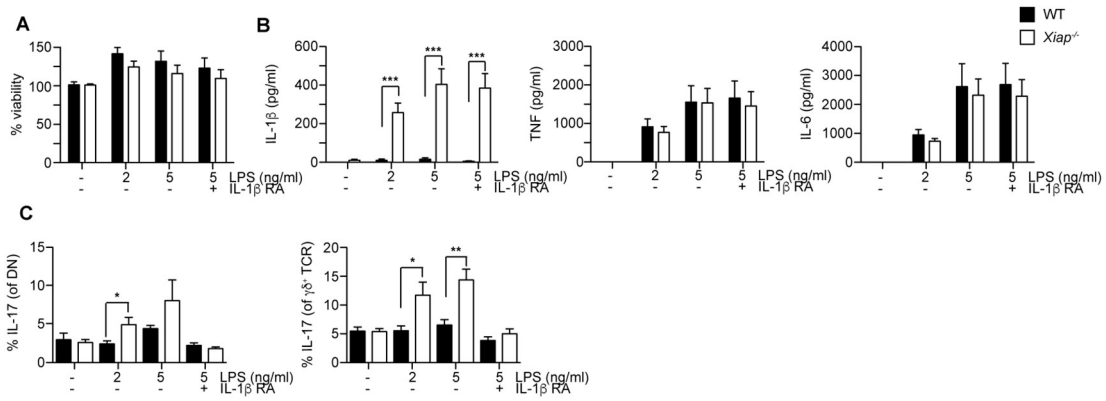


Figure 4. 13.: BMDMs show similar cytokine patterns like BMDCs without death and trigger IL-17 in coculture.

(A) Survival of WT and *Xiap*$^{-/-}$ BMDCs after 24h of LPS (2ng/ml, 5ng/ml, 10ng/ml)plus additional Anakinra (B) Secreted IL-1β, TNF and IL-6 of WT and *Xiap*$^{-/-}$ BMDCs after 24h of LPS (2ng/ml, 5ng/ml, 10ng/ml) plus additional Anakinra (C) Percentage of intracellular IL-17 induction in γδ^{+} and γδ^{-} T lymphocytes.

4.3.3.TNF as an indirect IL-17 regulator

To examine upstream factors that control inflammatory death and IL-1β secretion in Xiap$^{-/-}$ BMDCs, further experiments were conducted. As necroptosis is a form of highly inflammatory cell death, which results in release of DAMPs like IL-1β (S. J. Martin 2016), necroptotic triggers were examined.

It is known that necroptotic death can be induced downstream of TLRs as well as death receptors, such as TNF (Berghe, Hassannia, and Vandenabeele 2016), and therefore the initiating signal was investigated. To examine the impact of TNF loss on T cell differentiation, TNF-deficient mice were crossed with *Xiap*$^{-/-}$ mice. TLR activation of *Tnf*$^{-/-}$ and *Tnf/Xiap*$^{-/-}$ BMDCs revealed a complete dependence of inflammatory cell death downstream of the TNF receptor, as loss of TNF completely abolished death of BMDCs (Fig.4.14.A).

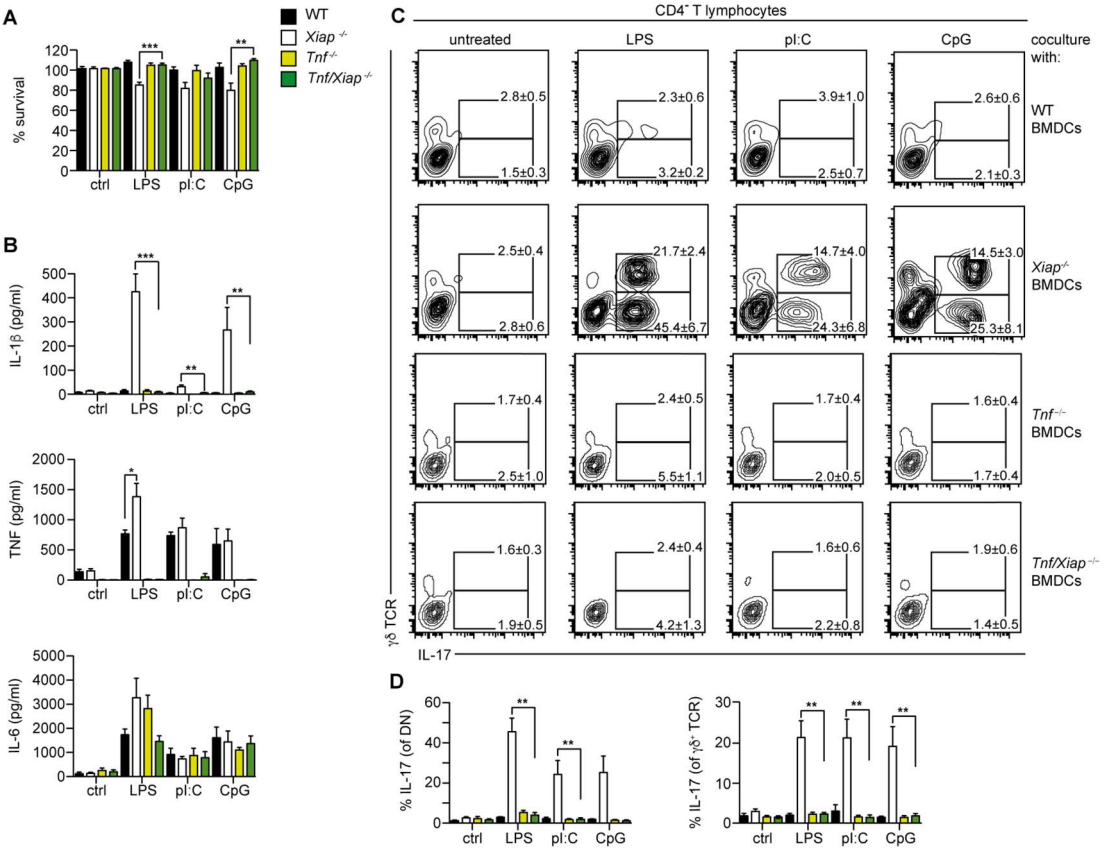


Figure 4. 14.: XIAP is involved in cell death induction downstream of the TNF receptor.

(A) Survival of WT, *Xiap*$^{-/-}$, *Tnf*$^{-/-}$ and *Tnf/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with LPS (2ng/ml), pI:C (10mg/ml), CpG (10nM). (B) Cytokines produced by WT, *Xiap*$^{-/-}$, *Tnf*$^{-/-}$ and *Tnf/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with LPS (2ng/ml), pI:C (10mg/ml), CpG (10nM). (C) Flow cytometric analysis of IL-17 in WT CD4^{-} T lymphocyte subsets after 5 days of coculture with treated (as described in 4.14.A) WT, *Xiap*$^{-/-}$, *Tnf*$^{-/-}$ and *Tnf/Xiap*$^{-/-}$ BMDCs. (D) Percentage of intracellular IL-17 induction in WT γδ and DN T cells of flow cytometric analysis depicted in 4.14.C.

Likewise, loss of death correlated with loss of IL-1β production in *Tnf/Xiap*$^{-/-}$ BMDCs, while IL-6 production remained unaltered (Fig.4.14.B). Using

Tnf/Xiap$^{-/-}$ BMDCs in the coculture setup, flow cytometric analysis revealed complete loss of IL-17 induction in T lymphocytes (Fig.4.14.C, 4.14.D).

Consequently, it can be concluded, that XIAP regulates cell death downstream of TNF and that induction of cell death does not occur via TLR stimulation, but rather is an effect of a positive feedback loop of self-produced TNF by the dendritic cells.

4.3.4. Shared Caspase 1 and Caspase 8 driven IL-1β processing

Since not only death was blocked after loss of TNF signaling but also IL-1β production, the source of IL-1β was further elucidate. Given that IL-1β is usually produced as an inactive pro-form, which can be cleaved into an active molecule by Caspase 1 (Thornberry et al. 1992), the regulation of this process during inflammatory cell death was assessed. Therefore, *Caspase 1/11* knockout mice were crossed with *Xiap*$^{-/-}$ mice. Treatment of *Caspase1/11/Xiap*$^{-/-}$ BMDCs with TLR ligands exhibited slightly less death then *Xiap*$^{-/-}$ BMDCs (Fig.4.15.A), while showing a significant reduction in IL-1β secretion (Fig.4.15.B). Although IL-1β was only detectable in reduced amounts, TNF and IL-6 production was unaltered (Fig.4.15.B).

Surprisingly, when the treated *Caspase1/11/Xiap*$^{-/-}$ BMDCs were used for a coculture setup, a similar IL-17 production, like with *Xiap*$^{-/-}$ BMDCs, could be seen (Fig.4.15.C, 4.15.D). Having a comparable amount of IL-17^{+} T cells after coculture, despite the reduced amounts of processed IL-1β, shows how sensitive innate T lymphocytes are to smallest changes in the cytokine environment around them.

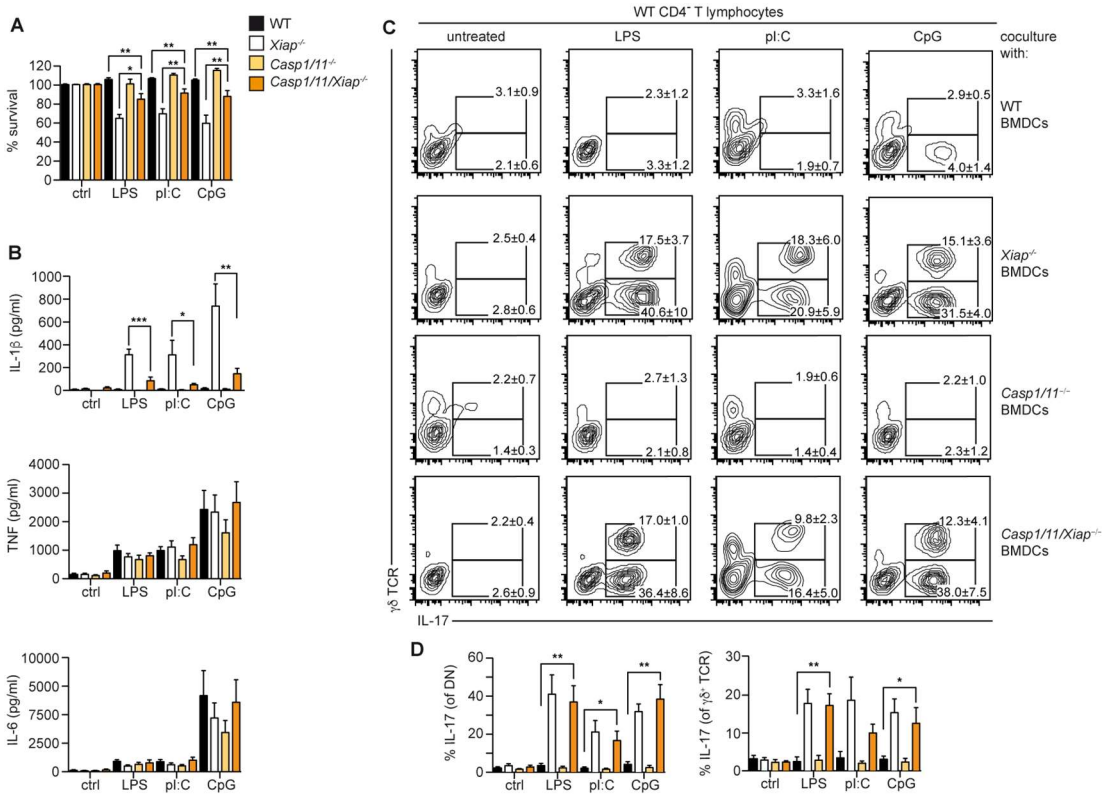


Figure 4. 15.: Loss of Caspase 1 does not influence IL-17 induction.

(A) Survival of WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with LPS (2ng/ml), pI:C (10mg/ml), CpG (10nM). (B) Cytokines produced by WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with LPS (2ng/ml), pI:C (10mg/ml), CpG (10nM). (C) Flow cytometric analysis of IL–17 in WT CD4^{-} T lymphocyte subsets after 5 days of coculture with treated (as described in 4.15.A) WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs. (D) Percentage of intracellular IL-17 induction in WT γδ and DN T cells of flow cytometric analysis depicted in 4.15.C.

Due to the circumstance that after Caspase 1/11 loss distinct levels of processed IL-1β were still sufficient to drive IL-17 induction in T lymphocytes, further experiments were conducted. According to recent publications not only the protease Caspase 1 can be involved in IL-1β processing, but also Caspase 8 (Moriwaki et al. 2015).

Therefore, BMDCs with a Caspase1/11 and XIAP double deficient background were treated with TNF and the Caspase 8 specific inhibitor Z-IETD-FMK (IETD). Identical to TLR treatment one could observe, that TNF treatment

resulted in more survival of *Caspase1/11/Xiap*$^{-/-}$ BMDCs in comparison to XIAP single knockouts (Fig.4.16.A). In contrast to TLR ligand induced cell death, TNF induced death was more pronounced. Additional treatment of dendritic cells with IETD did not influence viability. While loss of Caspase 1/11 already reduced IL-1β production around 60%, additional Caspase 8 inhibition further lowered IL-1β another 60%, leaving *Caspase1/11/Xiap*$^{-/-}$ BMDCs with almost no expression (Fig.4.16.B). TNF treatment barely induced IL-6 and this was independent of Caspase 8 (Fig.4.16.B). TNF treated *Xiap*$^{-/-}$ BMDCs were able to induce IL-17 comparable to TLR treatments (Fig.4.16.C, 4.16.D). This is further evidence that the induction of IL-17 in innate T lymphocytes is only dependent on IL-1β and not on other effects like TLR signaling. Even though IL-1β secretion was drastically reduced after genetic deletion of Caspase 1/11 in combination with chemical inhibition of Caspase 8, IL-17 induction in T cells was comparable (Fig.4.16.C, 4.16.D). This further emphasizes the sensitivity of innate-like T lymphocytes.

Inflammatory death accompanied by IL-1β processing can either be necroptosis or pyroptosis (Vasconcelos, Opdenbosch, and Lamkanfi 2016). As pyroptosis is dependent on Caspase 11 (Kayagaki et al. 2015), the next step was to investigate involvement of pyroptotic death, as viability increased after co-deletion of Caspase 1/11 and XIAP. Hence, WT, *Xiap*$^{-/-}$, *Ripk3/Xiap*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs after 8 and 24 hours of LPS treatment were immunoblotted and checked for Gasdermin D expression, which was recently described as the executioner of pyroptosis by forming membrane pores (Liu et al. 2016). Indeed, cleaved and thereby active Gasdermin D was visible after 8 hours in *Xiap*$^{-/-}$ BMDCs and was lost after co-deletion of RIPK3 and Caspase 1/11 (Fig.4.16.E). Moreover, co-deletion of RIPK3 and XIAP also abolished Caspase 8 activity.

In summary, XIAP inhibits inflammatory cell death downstream of the TNF receptor and negatively regulates IL-1β processing via Caspase 1 and Caspase 8 which results in IL-17 induction in T lymphocytes.

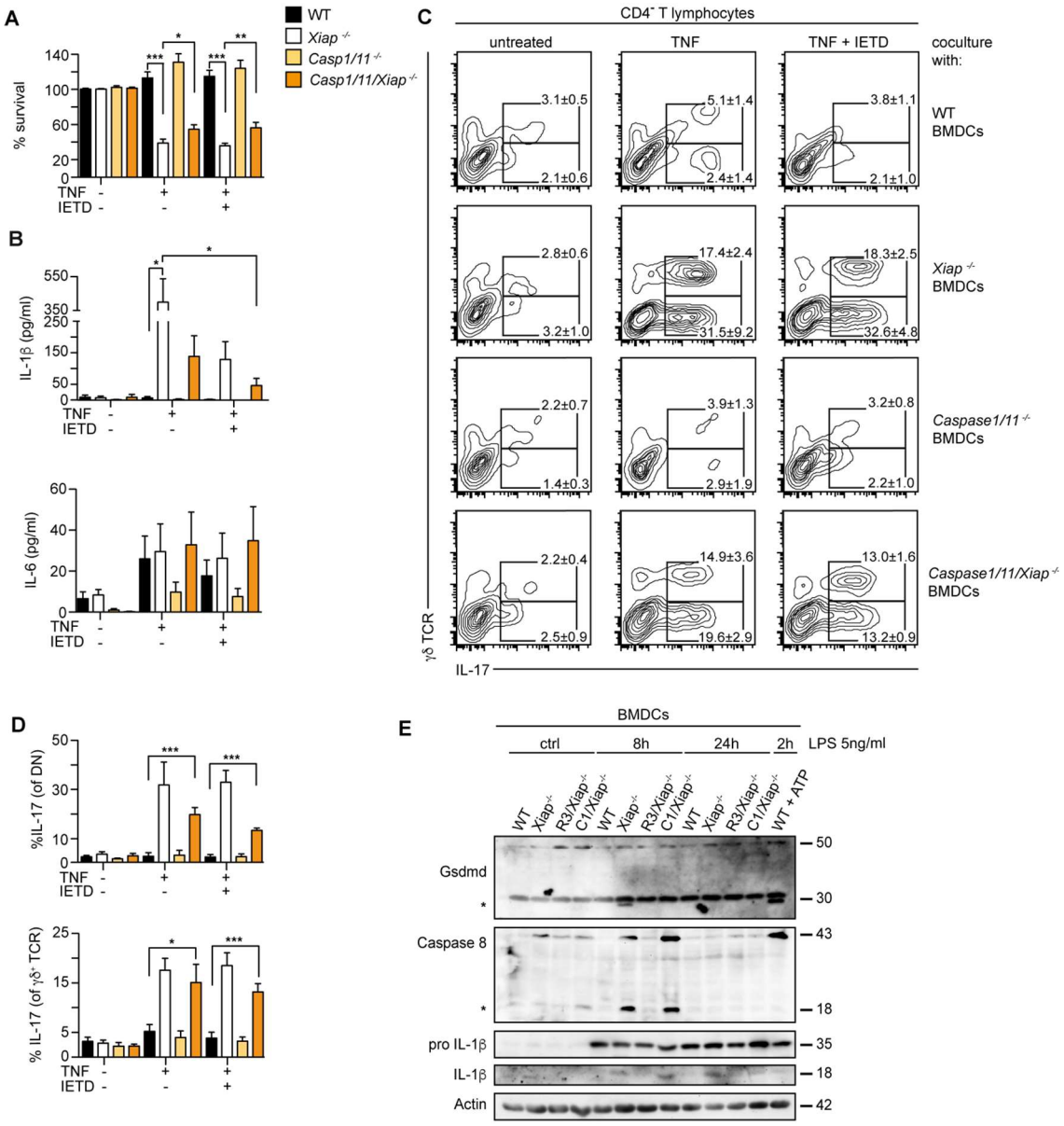


Figure 4. 16.: Caspase 1 and 8 together process IL-1β to facilitate IL-17.

(A) Survival of WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with TNF (100ng/ml) and additional IETD (10mM). (B) Cytokines produced by WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with TNF (100ng/ml) and additional IETD (10mM). (C) Flow cytometric analysis of IL–17 in WT CD4^{-} T lymphocyte subsets after 5 days of coculture with treated (as described in 4.15.A) WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs. (D) Percentage of intracellular IL-17 induction in WT γδ and DN T cells of flow cytometric analysis depicted in 4.15.C (E) Immunoblots of WT, *Xiap*$^{-/-}$, *Ripk3/Xiap*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs after LPS (5ng/ml) treatment for 8h and 24h. Last lane shows positive control with WT BMDC + LPS (2h) and ATP (30 min). * depicts cleaved Gasdermin D and Caspase 8

4.4. XIAP regulates inflammatory cell death dependent on RIPK3 but not in a MLKL dependent necroptotic setting

4.4.1. Inflammatory cell death execution is independent of RIPK1 kinase function

As mentioned before, inflammatory cell death can be accompanied by IL-1β processing either during pyroptosis or necroptosis. In the previous results section (4.3.4) a minor involvement of pyroptosis could be confirmed. Therefore, it was investigated whether the most studied form of programmed necrosis, called necroptosis occurred in the experimental setting. Necroptosis is described as being driven by RIPK1 and/or RIPK3 and MLKL. Consequently, the involvement of all three proteins during XIAP deficient signaling were investigated.

So far, the data indicates that XIAP restricts death induction and inflammasome activation downstream of the TNF receptor. Since the kinase RIPK1 is a critical signaling molecule downstream of the TNF receptor (Ofengeim and Yuan 2013), RIPK1 kinase activity has been reported to be essential for induction of necroptosis downstream of TNFR (He et al. 2011) and the fact that our work group has previously shown that RIPK1 ubiquitination is regulated by XIAP (Yabal et al. 2014), RIPK1 was a logical target. Thus, further investigations into the role of RIPK1 in inflammatory cell death and its consequences for T cell polarization were conducted.

Again WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs were treated with TNF in addition to Nec1s and GSK1728A, two RIPK1 kinase inhibitors. Inhibition of RIPK1 kinase activity with either of the chemicals, did not change the death rate in *Xiap*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs (Fig.4.17.A).

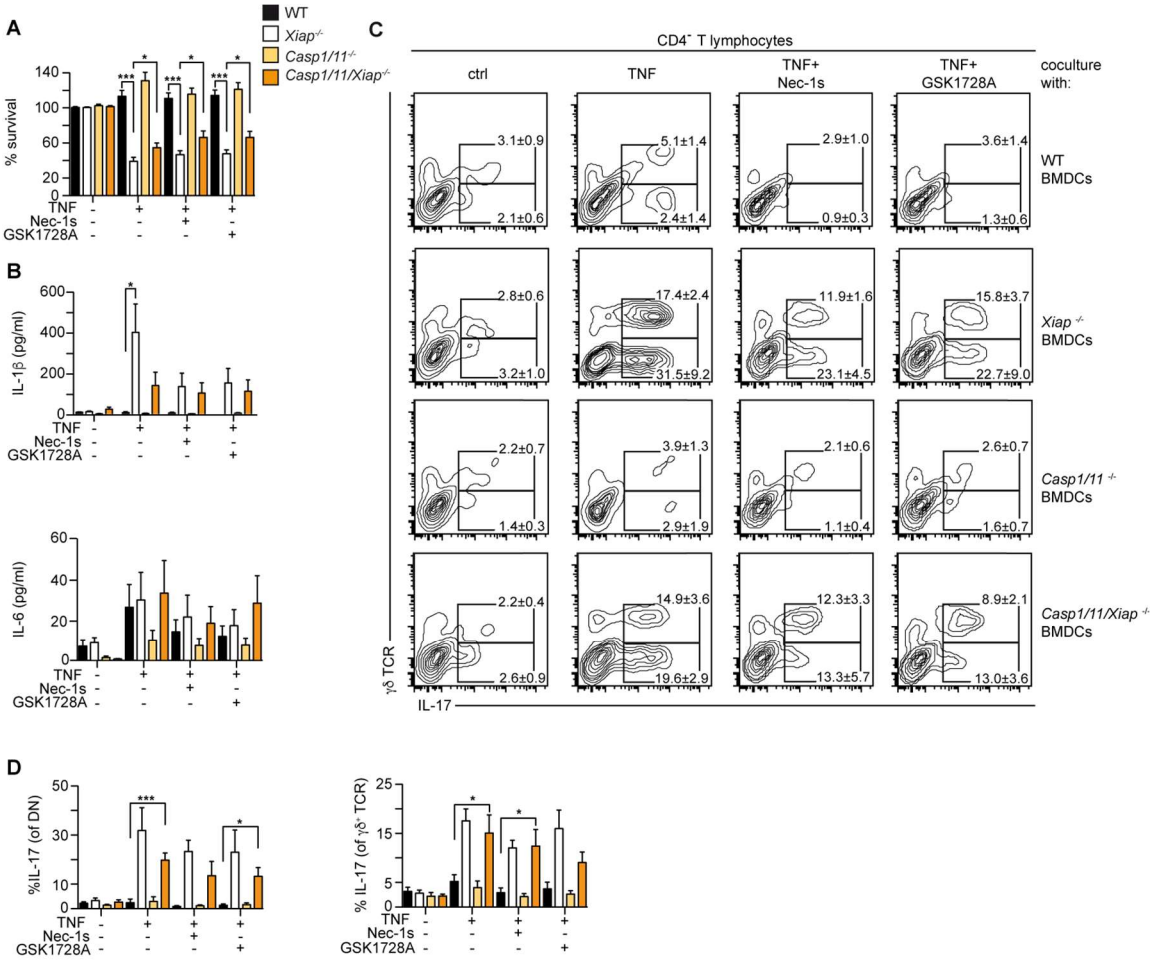


Figure 4. 17.: RIPK1 kinase influences Caspase 1 driven IL-1b processing but not execution of cell death.

(A) Survival of WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with TNF (100ng/ml) and additional Nec1s (30μM) or GSK1728A (10μM). (B) IL–1β and IL-6 in the supernatant of WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with TNF (100ng/ml) and additional Nec1s (30μM) or GSK1728A (10μM). (C) Flow cytometric analysis of IL-17 in WT CD4^{-} T lymphocyte subsets after 5 days of coculture with treated (as described in 4.17.A) WT, *Xiap*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs. (D) Percentage of intracellular IL-17 induction in WT γδ and DN T cells of flow cytometric analysis depicted in 4.17.C).

On the contrary, the inhibition of RIPK1 kinase activity seemed to have an effect on IL-1β processing instead. In *Xiap*$^{-/-}$ BMDCs IL-1β processing was lowered when RIPK1 kinase was inhibited and therefore IL-1β amounts were reduced to the levels usually processed by Caspase 8 (Fig.4.17.B). In contrast,

IL-1β magnitude was unaltered after kinase inhibition in *Caspase1/11/Xiap*$^{-/-}$ BMDCs, suggesting that Caspase 8 dependent IL-1β processing was independent of RIPK1 kinase activity. Again, TNF treatment barely induced IL-6 and this was not altered after RIPK1 kinase inhibition (Fig.4.17.B). As a consequence of incomplete IL-1β loss, T lymphocytes showed similar IL-17 induction after coculture with *Xiap*$^{-/-}$ and *Caspase1/11/Xiap*$^{-/-}$ BMDCs even after RIPK1 kinase inhibitor treatment (Fig.4.17.C, 4.17.D).
It can be concluded that in a context of XIAP loss driven inflammatory cell death, RIPK1 kinase function is not needed. Whether RIPK1 may instead work as a scaffold protein similar to its function in complex I downstream of the TNFR needs further evaluation. Moreover, RIPK1 kinase seems to have a critical role in transmitting signals form the TNF receptor to the Caspase 1 inflammasome.

4.4.2. XIAP regulates inflammatory cell death dependent on RIPK3

Previous experiments showed no involvement of RIPK1 kinase activity. Still the downstream effector molecule RIPK3 was examined, as RIPK3 has been described to be able to induce necroptosis downstream of the TNF receptor even in the absence of RIPK1 (Moujalled et al. 2013).

Therefore RIPK3 deficient mice were crossed with XIAP deficient mice and BMDCs were differentiated. After 24 hours of treatment *Ripk3*$^{-/-}$*Xiap*$^{-/-}$ BMDCs showed unaltered viability, identical to WT and *Ripk3*$^{-/-}$ BMDCs (Fig.4.18.A). With the loss of cell death in double deficient cells the expression of IL-1β was also completely abolished, while TNF and IL-6 remained unaltered (Fig.4.18.B). Moreover, induction of DN and γδ IL17^{+} T cells was completely abolished after coculture with *Ripk3*$^{-/-}$*Xiap*$^{-/-}$ BMDCs (Fig.4.18.C, 4.18.D).

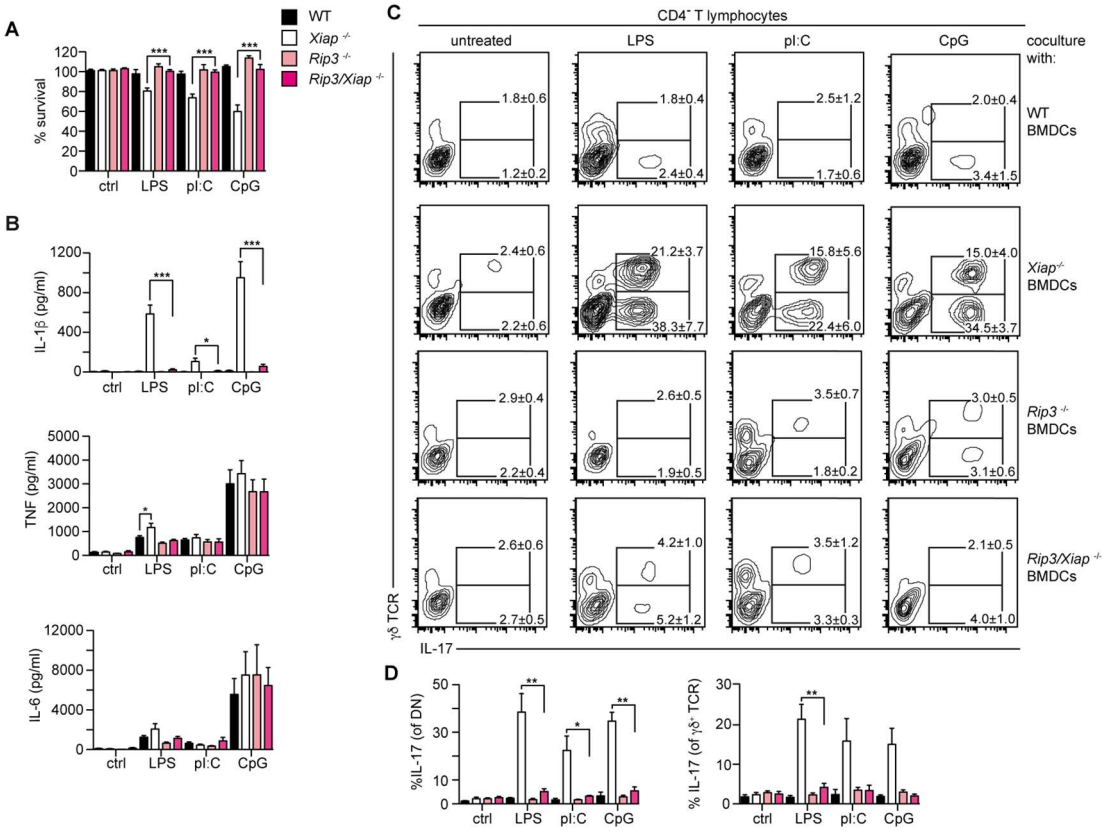


Figure 4. 18.: Co-deletion of RIPK3 and XIAP abolishes inflammatory cell death. (A) Survival of WT, *Xiap*$^{-/-}$, *Ripk3*$^{-/-}$ and *Ripk3/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with LPS (2ng/ml), pI:C (10mg/ml), CpG (10nM). (B) (A) IL–1β, TNF and IL-6 from the supernatant of WT, *Xiap*$^{-/-}$, *Ripk3*$^{-/-}$ and *Ripk3/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with LPS (2ng/ml), pI:C (10mg/ml), CpG (10nM). (C) & (D) Flow cytometric analysis and percentage of IL-17 in WT CD4^{-} T lymphocyte subset after 5 days of coculture with treated (as described in 4.18.A) WT, *Xiap*$^{-/-}$, *Ripk3*$^{-/-}$ and *Ripk3/Xiap*$^{-/-}$ BMDCs.

4.4.3. MLKL is dispensable for XIAP driven inflammatory death

Since RIPK3-dependent necroptosis results in phosphorylation of MLKL and subsequent pore formation (H. Wang et al. 2014), the dependence of XIAP driven cell death on MLKL was investigated. Hence, MLKL deficient mice were crossed with *Xiap*$^{-/-}$ mice, BMDCs differentiated and the impact of MLKL loss on T cell differentiation was determined.

TLR activation of *Mlkl/Xiap*$^{-/-}$ BMDCs revealed that loss of MLKL did not alter the viability of *Xiap*$^{-/-}$ BMDCs (Fig.4.19.A). Likewise, cytokine production was identical among *Mlkl/Xiap*$^{-/-}$ BMDCs and *Xiap*$^{-/-}$ BMDCs (Fig.4.19.B). Thus, T cell polarization was equal between T cells in coculture with activated *Mlkl/Xiap*$^{-/-}$ BMDCs and *Xiap*$^{-/-}$ BMDCs (Fig.4.19.C, 4.19.D), showing a strong increase in DN and γδ IL-17^{+} T cells.

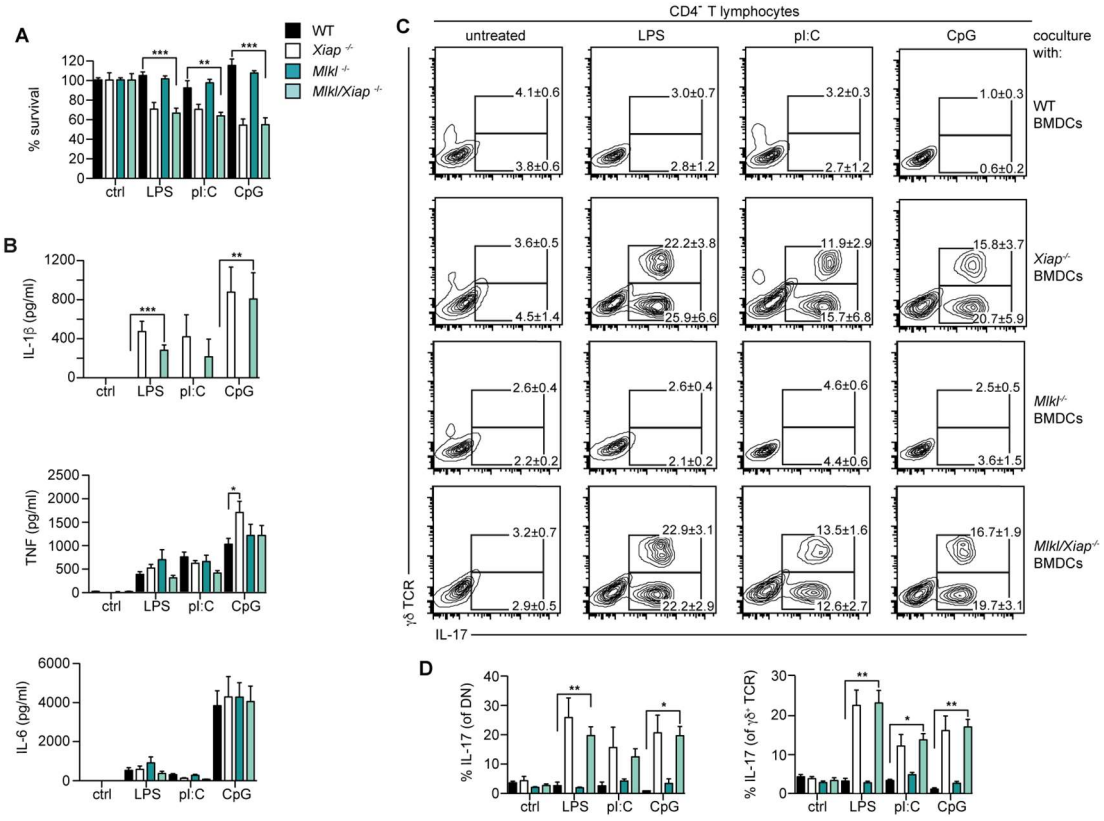


Figure 4. 19.: Loss of MLKL does not prevent induction of inflammation.
(A) Survival of WT, *Xiap*$^{-/-}$, *Mlkl*$^{-/-}$ and *Mlkl/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with LPS (2ng/ml), pI:C (10mg/ml), CpG (10nM). (B) Cytokines produced by WT, *Xiap*$^{-/-}$, *Mlkl*$^{-/-}$ and *Mlkl/Xiap*$^{-/-}$ BMDCs after 24h of stimulation with LPS (2ng/ml), pI:C (10mg/ml), CpG (10nM). (C) & (D) Flow cytometric analysis and percentage of IL-17 in WT CD4^{-} T lymphocyte subset after 5 days of co–culture with treated (as described in 4.19.A) WT, *Xiap*$^{-/-}$, *Mlkl*$^{-/-}$ and *Mlkl/Xiap*$^{-/-}$ BMDCs.

4.5. Loss of XIAP *in vivo* results in a heightened immune response to innate immunity triggers

To translate the described *in vitro* results into an *in vivo* context and therefore give them a more clinical relevance, three different experimental approaches were conducted. These include the intraperitoneal injections of LPS, infections with Citrobacter rodentium and two different settings of Graft versus Host disease (GvHD). All of these experimental setups include triggers for innate immunity, similar to the TLR ligands used in the coculture setup and reactions of the immune system, especially the T lymphocytes were monitored.

4.5.1. Intraperitoneal LPS triggers XIAP driven hyperinflammatory responses

XLP-2 disease is associated with up to 90% hemophagocytic lymphohistiocytosis (HLH) (Rigaud et al. 2006) which is often linked to a viral infection as a trigger for disease outbreak. Anyhow viral infections are not the only type of infection documented to occur during XIAP deficiency. Apart from others, also bacterial burdens like *H. influenzae* have been documented in XLP-2 patients (Schmid et al. 2011).

To transfer the *in vitro* data into an *in vivo* setting, intraperitoneal LPS injections (200μg per mouse) were performed as a basic model of infection. After 4 hours, immune responses in WT and *Xiap*$^{-/-}$ mice were analyzed by flow cytometry. Thus, amounts of γδ, iNKT17, DN and CD4^{+} T lymphocytes in the mesenteric lymphnodes (ms LN) and the spleen, as well as their respective intracellular IL-17 expression were measured. FACS analysis showed a significant increase of IL-17 expression after 4 hours in γδ T lymphocytes located in the mesenteric lymphnodes of *Xiap*$^{-/-}$ mice, while IL-17 was only slightly increased in the spleen with unaltered γδ^{+} amounts in both organs (Fig.4.20.A). Moreover, an elevated recruitment of iNKT cells in both organs with an increased IL-17 production was detected (Fig.4.20.B). There were no

changes measurable in the recruitment or the IL-17 expression of CD4⁺ or DN T lymphocytes (Fig.4.20.C, 4.20.D). It is known from the literature, that an

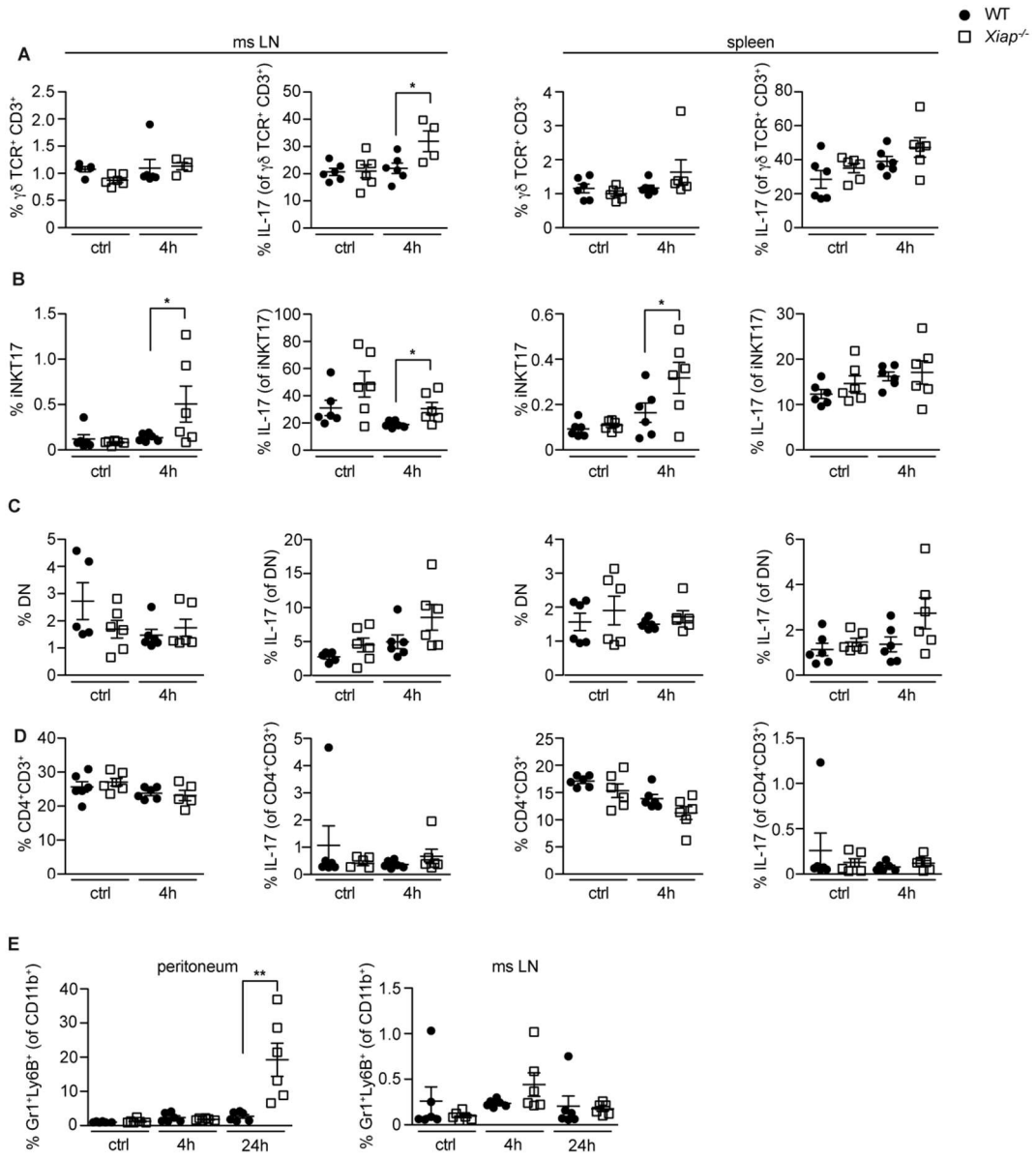


Figure 4. 20.: Intraperitoneal LPS induces recruitment of innate cell subsets and IL-17 expression in *Xiap*$^{-/-}$ mice.

Each dot represents a mouse and error bars represent mean (±SEM). Percentage of T lymphocyte influx into the mesenteric lymphnodes (ms LN) and spleen as well as their respective intracellular IL-17 expression after 4h of intraperitoneal injection of LPS (200μg/mouse). (A) γδ T cells (B) iNKT17 T cells (C) DN T cells (D) CD4⁺ T cells (E) Percentage of neutrophil influx into the peritoneum und mesenteric lymphnodes after 4h and 24h of intraperitoneal injection of LPS (200μg per mouse).

inflammation driving cell subset to be recruited by IL-17 are neutrophils (Witowski et al. 2000). Hence, it was tested if elevated IL-17 levels in the experimental mice were sufficient to recruit neutrophils. FACS analysis revealed a significant increase of neutrophils after 24 hours in the peritoneum of *Xiap*$^{-/-}$ mice and additionally a minor increase in the mesenteric lymphnodes after 4 hours in *Xiap*$^{-/-}$ mice (Fig.4.20.E).

In addition to flow cytometric analysis, cytokines locally in the peritoneum and systemically in the serum were measured. After 4 hours IL-1β, as well as other inflammatory cytokines like IL-17, TNF and IFNγ were significantly increased in the peritoneum of *Xiap*$^{-/-}$ mice (Fig.4.21.A). This translated also in a systemic setting, showing significantly elevated IL-1β and IL-17, as well as increased TNF and IFNγ in the serum of *Xiap*$^{-/-}$ mice 4 hours after being challenged (Fig.4.21.B). This effect was only visible shortly after LPS challenge and vanished after 24 hours in the peritoneum as well as in the serum (data not shown), indicating that disease initiating or supporting inflammatory flares may only be visible in a defined short time window in XLP-2 patients.

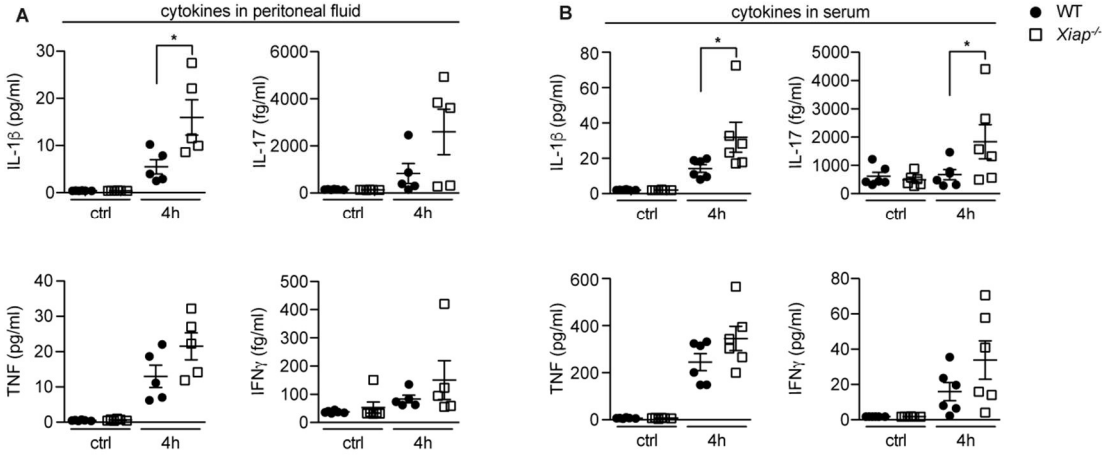


Figure 4. 21.: Loss of XIAP triggers local and systemic inflammation after LPS challenge.

T cell intracellular cytokines (A) and (B) Cytokines in peritoneal fluid and the serum of WT and *Xiap*$^{-/-}$ mice after intraperitoneal injection of LPS (200μg/mouse) after 4h, Each dot represents a mouse and error bars represent mean (±SEM).

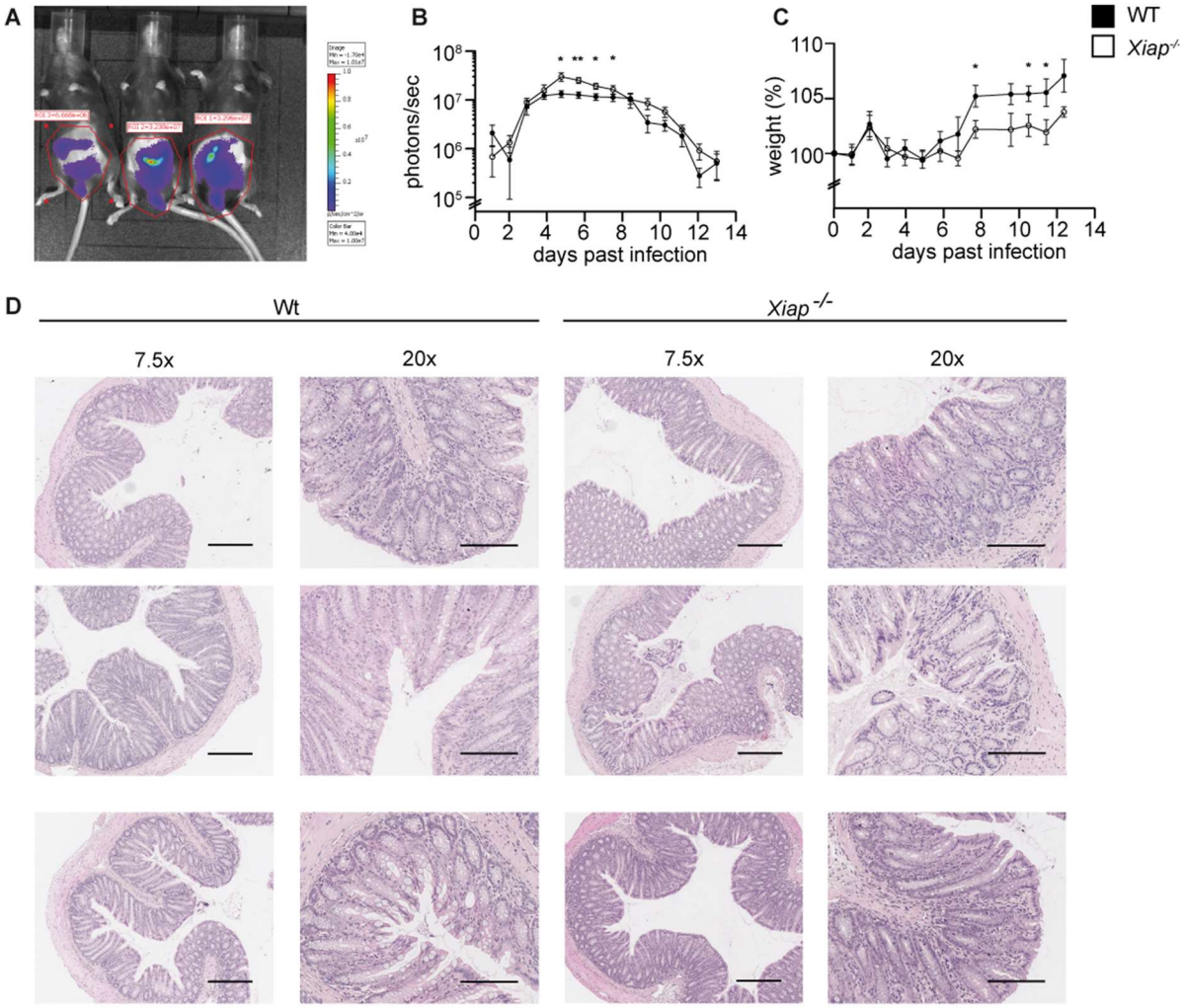
In summary, the data shows that the phenotype of TLR ligand triggered IL-17 induction *in vitro* is transferable into an *in vivo* setting. This indicates that evaluation of IL-17 in XLP-2 patients may be a clinical parameter to be tested.

4.5.2. XIAP deficiency drives *Citrobacter rodentium* susceptibility

Scientific literature reports that around 25-30% of XLP-2 patients can suffer from IBD symptoms (Aguilar and Latour 2015; Latour and Aguilar 2015). IBD can also occur before any other indication or be the only manifestation at all (Yvonne Zeissig et al. 2014). Up to now, IBD symptoms in XIAP deficient patients were associated with the defect in NOD2 signaling due to the absence of XIAP (Damgaard et al. 2013). IBD disease progression might be further enhanced by deregulated inflammatory cell death disrupting the epithelial barrier and inflammation being fueled by IL-17, a cytokine closely connected to IBD pathogenesis (Catana et al. 2015). Therefore, a mouse model system with Citrobacter rodentium infection was used, which is known to mimic IBD symptoms (J. W. Collins et al. 2014). Besides, C. rodentium infections are known to be tightly regulated by IL-1β, which keeps the balance between fighting the infection and overshooting immune reactions with barrier disruption as a consequence (Alipour et al. 2013). Experimental procedures were executed in a collaboration by Vera Kitowski from AG Hildner at the first medical department in the medical clinic Erlangen. Experimental setup, data analysis, histology and interpretation were performed by the doctoral candidate.

Prior to infection WT and *Xiap*$^{-/-}$ Bl6 mice were cohoused for at least 5 weeks to ensure microbial assimilation, as differences in the microbial flora may result in different experimental outcomes (JW Collins 2014 Nat Rev). To induce colitis, mice were subject to oral gavage with a Citrobacter rodentium strain capable of bioluminescence. Severity of infection was tracked daily by luminescence monitoring (Fig.4.22.A). Bioluminescence tracing revealed an increased bacterial burden in *Xiap*$^{-/-}$ mice, with a peak in infection between day 5 and day 8 (Fig.4.22.B). The increased severity of the course of infection

was further reinforced by the worse weight recovery in XIAP deficient mice, which started to differ from WT mice after the peak of infection (Fig.4.22.C). Although bacterial burden was aligned between WT and *Xiap*$^{-/-}$ mice at the end of infection, histological evaluation of the colon revealed still major differences at day 14. 7,5x magnification of histological slides after H&E staining, shows hyperplasia with denser packing of the colonic mucosa and crypt elongation in *Xiap*$^{-/-}$ mice (Fig.4.22.D). 20x magnifications additionally reveals a loss of goblets cells and an increased lymphocyte infiltration in XIAP deficient mice (Fig.4.22.D). To further analyze differences between genotypes, mice were sacrificed at day 8, the peak of the Citrobacter rodentium infection and cells of the lamina propria and intraepithelial compartment from ileum and colon were purified.



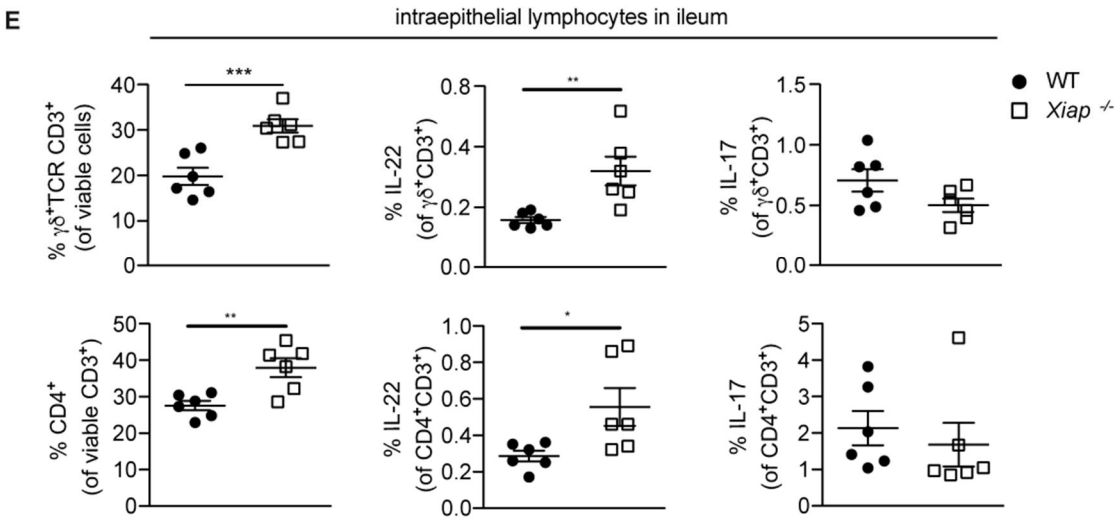


Figure 4. 22.: Loss of XIAP leads to increased Citrobacter rodentium susceptibility.

(A) Exemplary picture taken of narcotized mice for bioluminescence imaging and measurement (B) Course of Citrobacter rodentium infection of WT and *Xiap*$^{-/-}$ mice diagrammed as a graph of mean (+/– SEM) photons/sec per day of infection (C) Weight curve of WT and *Xiap*$^{-/-}$ mice during infection (D) H&E stainings of WT and *Xiap*$^{-/-}$ mice (3 each) at day 14 with 7,5x and 20x magnification. Black bars indicate 300μm (7,5x magnification) and 400μm (20x magnification) (E) Flow cytometric analysis of intraepithelial lymphocytes of WT and *Xiap*$^{-/-}$ mice at day 8 of Citrobacter rodentium infection with represented CD4$^+$ and γδ T cells and their individual intracellular expression of IL-22 and IL-17.

Flow cytometric analysis showed no differences in between genotypes with regards to lamina propria of colon and ileum or the colonic intraepithelial lymphocytes (data not shown). However, in the intraepithelial lymphocyte compartment of the ileum, a significant increase in CD4$^+$ and γδ T cells was detectable in comparison to WT mice (Fig.4.22.E). Though no change in CD4$^+$IL-17$^+$ or γδ$^+$IL-17$^+$ was traceable, a significant increase in IL-22 expression in both CD4$^+$ and γδ T cells was registered.

Summarized, XIAP deficient mice clearly are more susceptible to Citrobacter rodentium infections including increased bacterial burden, poor weight recovery and heightened inflammation in the colon and ileum. Although this affected phenotype did not seem to correlate with increased IL-17, an elevated IL-22 paralleled the burden of *Xiap*$^{-/-}$ mice.

4.5.3. Loss of XIAP in donor cells does not influence GvHD induction or progression in recipient mice

The only curative treatment option for XLP-2 disease is a hematopoietic stem cell transplantation (HSCT), a procedure that is often associated with high mortality rates (Marsh et al. 2013). Moreover, a common complication after transplantation is the development of a sever graft versus host disease (GvHD) (Marsh et al. 2013). GvHD results from conditioning initiating a cytokine storm and an inflammatory reaction in APCs, which activate donor derived T lymphocytes and thus an alloreactive graft (donor) versus host reaction (Ferrara et al. 2009). With regard to the collective *in vitro* data, it was postulated, that conditioning of recipients results in increased necroptosis, hyperinflammation and therefore a stronger development of GvHD in case of XIAP deficiency. On these grounds, the third experimental setup was a mouse model system for GvHD.

In the first setup, the goal was to determine whether XIAP or RIPK3 dependent signaling pathways in donor cells played a role after the transplantation into the host for GvHD induction. In the experimental procedure bone marrow (BM) and $CD4^+/CD8^+$ T cells from the spleen were isolated from donors with a WT, *Xiap*$^{-/-}$, *Rip3*$^{-/-}$, *Rip3/Xiap*$^{-/-}$ genetic background. (Fig.4.23).

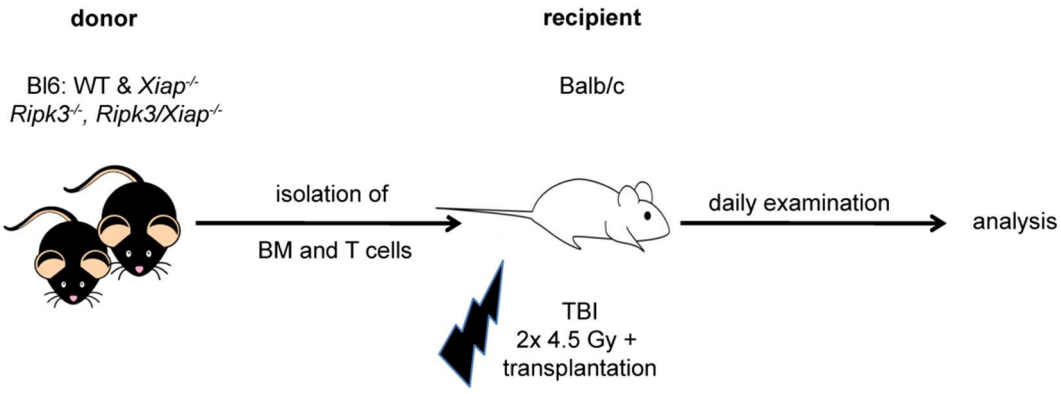


Figure 4. 23.: Experimental setup with Bl6 donors and Balb/c recipients.
After isolation of bone marrow (BM) and T cells from donors with different genetic backgrounds, conditioned Balb/c mice were transplanted and examined daily for signs of GvHD including weight tracking, skin GvHD.

After conditioning of Balb/c mice with a split dose irradiation, the mixture of BM and T cells was transplanted in a blinded manner and from thereon recipients were examined daily.

To ensure an equal premise, the contents of Treg cells within the transplanted $CD4^+$ subset was determined. Treg cells are described to be able to dampen GvHD severity (Edinger et al. 2003), but all four different donors had comparable amounts of Treg lymphocytes (Fig.4.24.A). Flow cytometric analysis differentiated H2kb and H2kd, Bl6 and Balb/c specific MHC-I-like cell surface markers, and verified successful engraftment at day 14 after transplan-

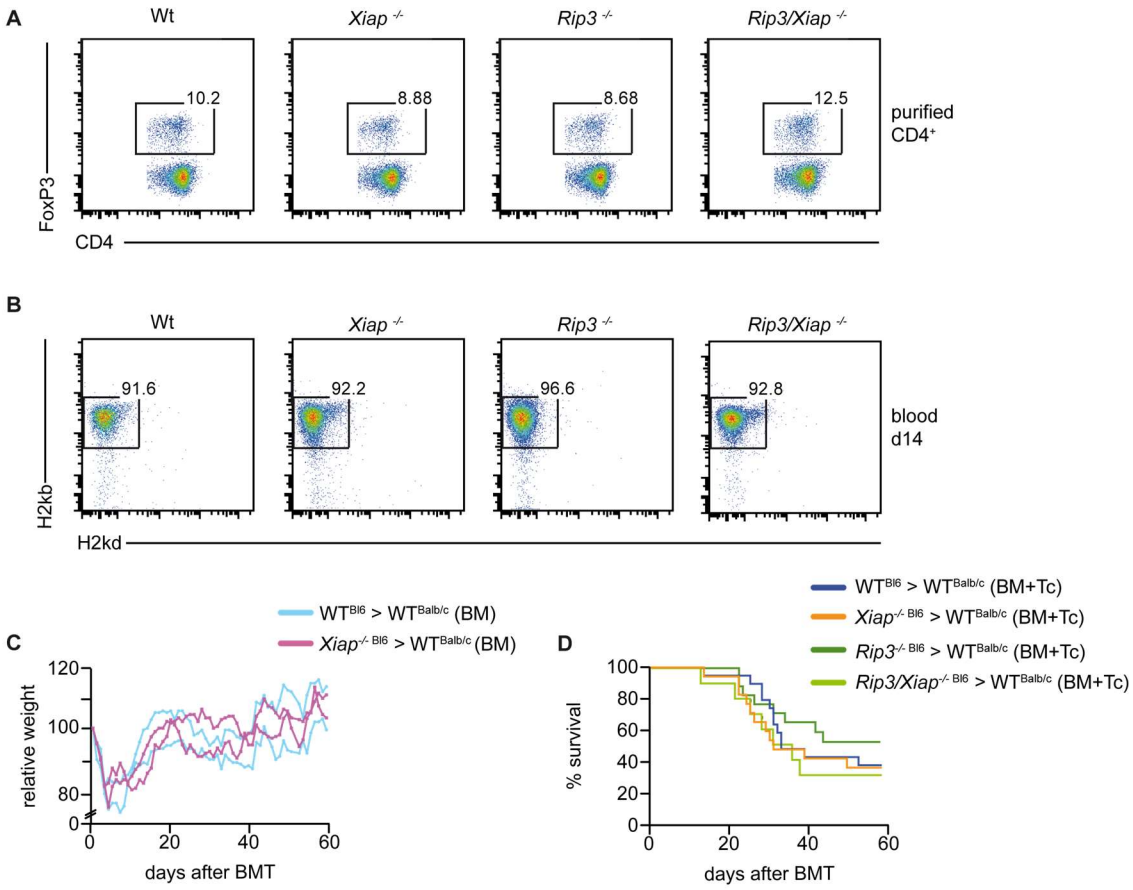


Figure 4. 24.: XIAP and RIPK3 driven pathways in donor cells do not influence GvHD induction and progression.

(A) Exemplary flow cytometric analysis of purified donor $CD4^+$ cells for FoxP3 and CD4. (B) Exemplary flow cytometric analysis of blood cells of recipients at day 14 for Bl6 (H2kb) and Balb/c (H2kd) specific markers. (C) Relative weight curves over time after transplantation of two WT and two *Xiap*$^{-/-}$ BM transplanted recipients. (D) Kaplan–Meier survival curve after transplantation of WT (n=18), *Xiap*$^{-/-}$(n=17), *Rip3*$^{-/-}$ (n=17) or *Rip3/Xiap*$^{-/-}$ (n=10) BM + T cells transplanted recipients.

tation (Fig.4.24.B). Transplantation of bone marrow cells alone resulted in no induction of GvHD, indicated by the successful weight recovery and increase over the observation period (Fig.4.24.C). Tracking GvHD induced mortality over time displayed no difference in the survival of Balb/c mice reconstituted either with Bl6 WT, *Xiap*$^{-/-}$, *Rip3*$^{-/-}$ or *Rip3/Xiap*$^{-/-}$ cells. This indicates that neither XIAP nor RIPK3 play a role in donor derived cells.

4.5.4. XIAP deficiency in recipients results in worse GvHD prognosis and is dependent on myeloid cells

For quite some time after conditioning, a chimerism of donor-derived and conditioning resistant recipient APCs can be found in recipients (Shlomchik 2007). Although donor APCs are required for a full blown GvHD, also recipient APCs are necessary and sufficient for GvHD (Shlomchik 2007). It was also shown, that early IL-1β production was recipient APCs derived, whereas only later donor derived IL-1β production supported GvHD induction (Jankovic et al. 2013). As it was shown in previous experiments that loss of XIAP in donor derived cells had no impact on GvHD induction or development (Fig.4.24.D), it was tested whether XIAP deficiency in recipient cells had any influence on GvHD.

Prior to transplantation WT and *Xiap*$^{-/-}$ mice were cohoused to ensure microbial assimilation. Bl6 mice were irradiated with increased intensity (split dose of 2x5,5Gy), as Balb/c mice are more susceptible to myeloablative conditioning (Schroeder and DiPersio 2011). After conditioning, BM and T cells from Balb/c donors were transplanted blinded into WT and *Xiap*$^{-/-}$ mice and recipients were controlled daily for signs of GvHD (Fig.4.25).

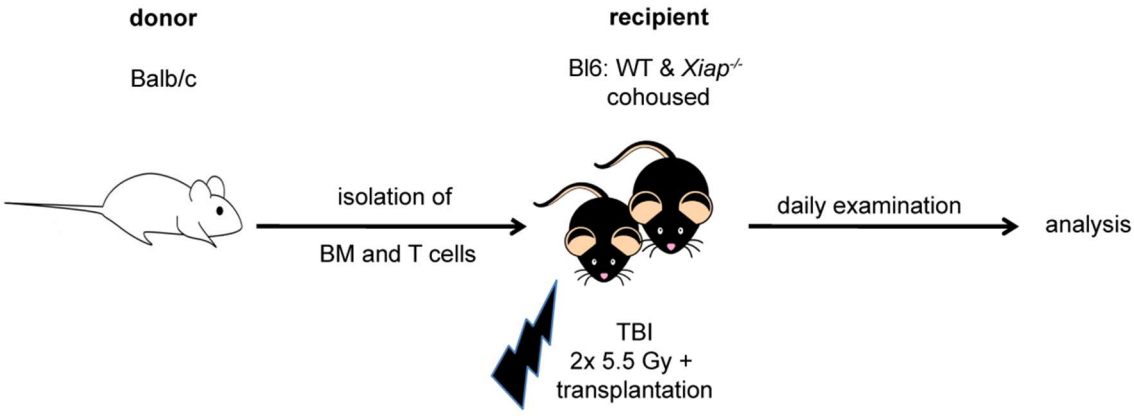


Figure 4. 25.: Experimental setup with Balb/c donors and Bl6 recipients.
After isolation of bone marrow (BM) and T cells from Balb/c donors, conditioned and cohoused Bl/6 mice were transplanted and examined daily for signs of GvHD including weight tracking, skin GvHD.

One sign of GvHD induction is diarrhea resulting in weight loss. Therefore, weight of recipient mice was controlled daily. The initial setup for GvHD induction was based on personal experience from a supporting lab, having used $5x10^6$ T cells for GvHD induction in the same mismatch model before. Relative weight curves of the mice show a strong induction of GvHD between day 13 and 18 and all mice had to be sacrificed due to severe weight loss before day 30 (Fig.4.26.A). As the severity of GvHD induction depends on dose and type of T cells, intensity of conditioning, the degree of mismatch and environmental pathogens (Schroeder and DiPersio 2011), a second attempt with reduced T cells of only $0,5x10^6$ was started. Identical to the first attempt a few mice died before day 10. Two *Xiap*$^{-/-}$ mice died of anemia around day 30 and only one *Xiap*$^{-/-}$ mouse had to be sacrificed due to GvHD (Fig.4.26.B). Due to this low induction of GvHD rate a third attempt was done. This third experiment included $1,0x10^6$ transplanted T lymphocytes. Excluding early deaths, GvHD induction of 50% was considered a successful induction rate (Fig.4.26.C). But like in both previous experiments, independent of T cells dose and genotype of recipient a high mortality before day 10 was recorded. Flow cytometric analysis could exclude graft failure as the reason for death, showing

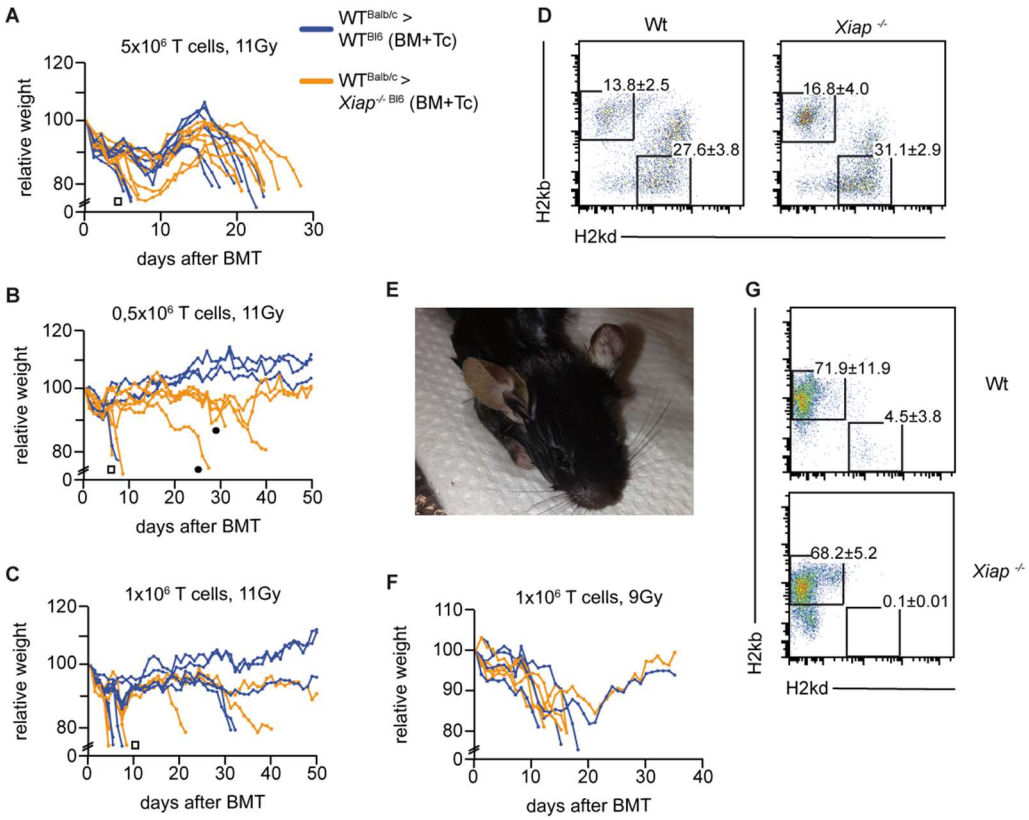


Figure 4. 26.: Adjustment of GvHD inducing conditions with Bl6 recipients.
(A) Relative weight curves of WT (n=9) and *Xiap*$^{-/-}$ (n=10) mice post transplantation with 5,0x10^6 T cells. (B) Relative weight curves of WT (n=4) and *Xiap*$^{-/-}$ (n=7) mice post transplantation with 0,5x10^6 T cells. (C) Relative weight curves of WT (n=8) and *Xiap*$^{-/-}$ (n=8) mice post transplantation with 1,0x10^6 T cells. (D) Flow cytometric analysis of bone marrow cells for Bl6 (H2kb) and Balb/c (H2kd) markers at time of death. (E) Examination of marked facial swelling. (F) Relative weight curves of WT (n=8) and *Xiap*$^{-/-}$ (n=8) mice post transplantation with 1,0x10^6 T cells after reduced irradiation of 9Gy. (G) Flow cytometric analysis of bone marrow cells for Bl6 (H2kb) and Balb/c (H2kd) markers at time of death after 9Gy irradiation.

□ indicates early death unrelated to GvHD induction

● indicates graft failure and anemia as reason for death

a mixed chimerism of donor and recipient cells in the bone marrow at time point of death (Fig.4.26.D). After adjusting the right T cell dose, the phenotype of early, GvHD unrelated, death was examined. Close surveillance of mice after transplantation revealed, that mice which succumbed to “early death phenomenon” within hours, just showed symptoms shortly before death. These included a sudden weight drop in affected mice (Fig.4.26.A/B/C),

lethargy, hunched postures and around 50% of mice exhibited facial swelling (Fig.4.26.E). In 2008, Duran-Struuck and colleagues described differences between mouse strains and their susceptibility towards irradiation (Duran-Struuck et al. 2008). They observed that strain dependent radiation sensitivity lead to increased gastrointestinal damage, being so severe that overwhelming bacteremia was the main cause of death. Due to comparable symptoms, bacteremia was postulated to be the reason for death in the early phase after transplantation. To circumvent the “early death “phenotype, irradiation was reduced to a split dose of 4,5Gy, similar to Balb/c recipients. Radiation reduction completely eliminated early deaths, but unfortunately lead to high mortality after day 10 (Fig.4.26.F) and almost 100% graft failures (fig.4.26.G).

As reduced radiation did result in a high incidence of graft failures, the second option was to reduce bacterial load in the gut to avoid sepsis. Administration of antibiotics is a common pretreatment also done in human patients to tackle infections, especially in the neutropenic period after transplantation (LAROCCO and BURGERT 1997). Therefore, antibiotics were administered via drinking water two days prior to transplantation and were continued on till day 10. As environmental pathogens are one influencing factor for the severity of GvHD, it was not surprising that reduction of bacterial load resulted in a strongly diminished phenotype in WT mice. After initial weight loss WT mice recovered, while *Xiap*$^{-/-}$ mice started losing weight at around day 30 with a steady decline (Fig.4.27.A).

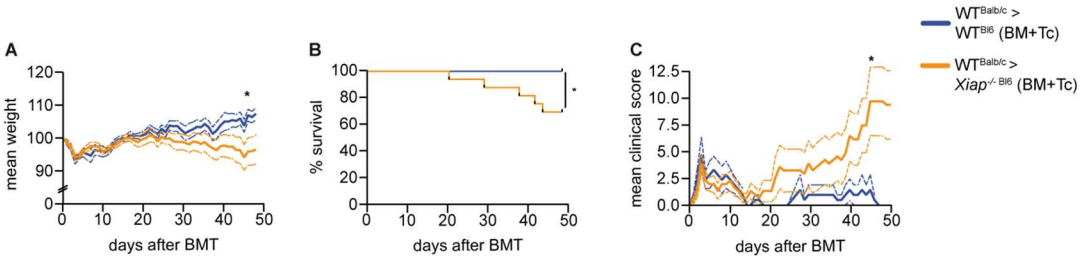


Figure 4. 27.: *Xiap*$^{-/-}$ mice are more prone to develop GvHD.
(A) Mean weight curve of WT (n=11) and *Xiap*$^{-/-}$ (n=16) mice after transplantation. (B) Kaplan–Meier survival curve of WT (n=11) and *Xiap*$^{-/-}$ (n=16) mice after transplantation. (C) Mean clinical score of WT (n=11) and *Xiap*$^{-/-}$ (n=16) mice after transplantation.

This translated into a significant difference in survival, where only *Xiap*$^{-/-}$ mice died due to GvHD (Fig.4.27.B). Moreover, a clinical score of pooled GvHD symptoms verified how burdened *Xiap*$^{-/-}$ mice were (Fig.4.27.C).

To get a better understanding of the mechanisms behind the differences in GvHD severity between WT and *Xiap*$^{-/-}$ mice, a time point analysis was conducted. Day 8 was chosen for analysis, due to the influence of *Xiap*$^{-/-}$ recipient cells early on in GvHD induction. The gastrointestinal tract has been shown to play a major role in amplification of systemic disease, where gut damage triggers the cytokine storm initiating GvHD (Hill and Ferrara 2000). Consequently, cellular composition in the colon and ileum was investigated. Flow cytometric analysis of the lamina propria (LP) as well as the intraepithelial compartment (IEC) revealed an increase of donor CD4^{+} and CD8^{+} in *Xiap*$^{-/-}$ guts (Fig.4.28.A). A similar pattern of donor T cell accumulation in *Xiap*$^{-/-}$ mice was true for the ileum (Fig.4.28.B). A more specific stain, including cytokine detection, exhibited that donor and recipient CD4^{+} T cells expressed significant less IL-17, while at the same time producing significant more IFNγ in the lamina propria of the colon (Fig.4.28.C). Evaluation of recipient $\gamma\delta$ T lymphocytes in the LP and IEC of the ileum mirrored the phenotype of increased IFNγ (Fig.4.28.D). That the observed cytokine pattern translated into a systemic context, was proven by cytokine detection in the serum at time of death. While increased amounts of IL-1β, IFNγ and TNF were detectable, IL-17 was significantly reduced in *Xiap*$^{-/-}$ mice (Fig.4.28.E).

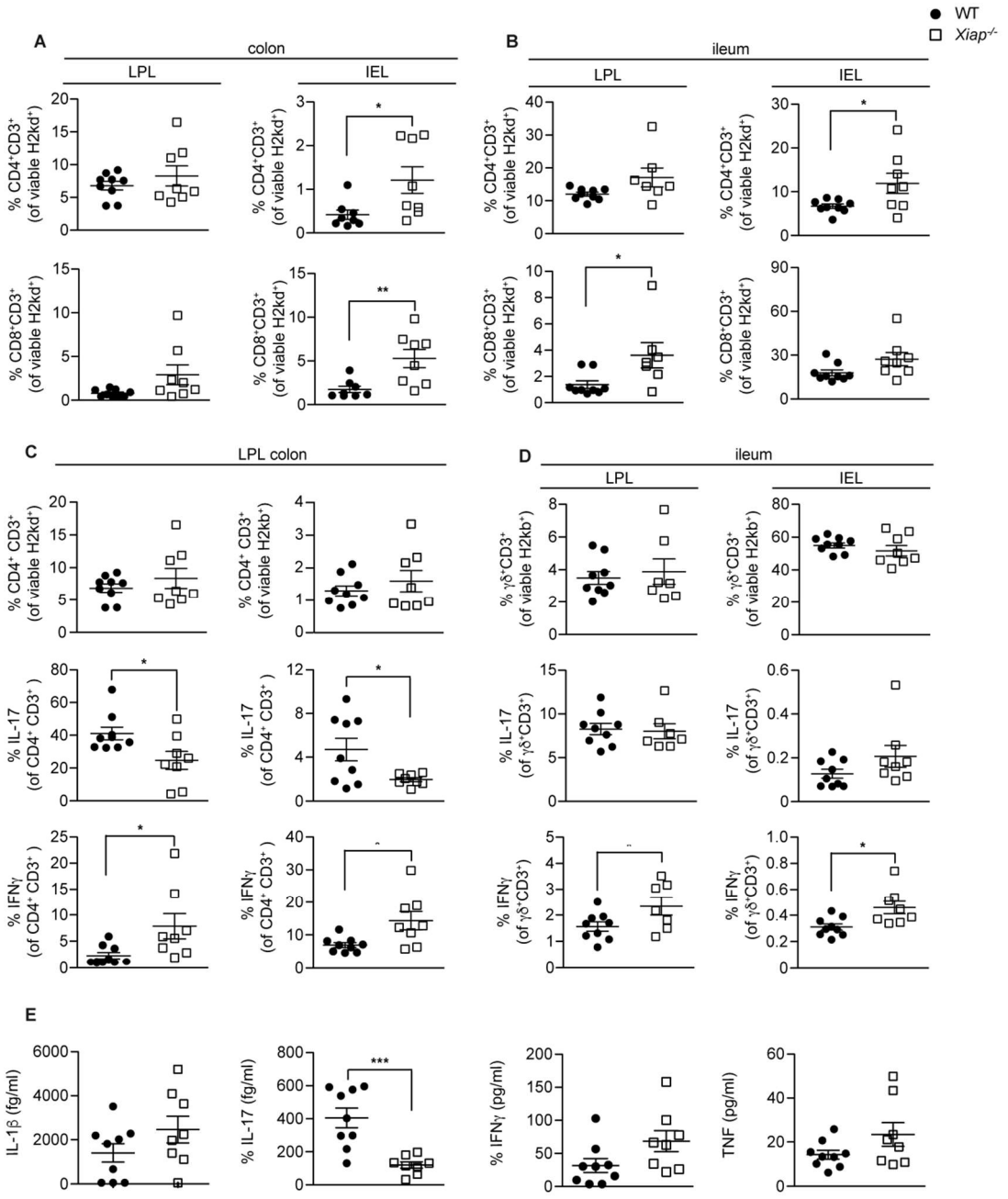


Figure 4. 28.: GvHD induction in Xiap$^{-/-}$ mice shows increased donor T cell accumulation and IFNγ production.

Each dot represents one mouse. All data obtained at day 8 past GvHD induction. (A) Flow cytometric analysis of WT and *Xiap*$^{-/-}$ mice colon, distinguishing between lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) infiltrated CD4$^+$ and CD8$^+$ donor cells. (B) Flow cytometric analysis of WT and *Xiap*$^{-/-}$ mice ileum, distinguishing between lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) infiltrated CD4$^+$ and CD8$^+$ donor cells. (C) Recipient and donor CD4$^+$ T cells and their intracellular expression of IFNγ and IL-17 in the lamina propria of the colon. (D) Recipient γδ T cells and their intracellular expression of IFNγ and IL-17 in LP and IEC of the ileum. (E) Cytokine levels in the serum of mice.

To examine which XIAP deficient cells drive increased GvHD, an experimental setup involving bone marrow chimeras was established. As *in vitro* experiments showed a strong phenotype dependence on dendritic cells and their inflammatory death, it was postulated that this might likewise be a driving factor in XIAP deficient GvHD induction. Therefore, WT Bl6 mice were irradiated and then transplanted with bone marrow from either WT Bl6 mice or *Xiap*$^{-/-}$ Bl6 mice (Fig.4.29.A). This resulted in chimeras where WT Bl6 mice had an exchanged hematopoietic compartment derived from donor bone marrow, either WT hematopoietic cells ($WT^{BL6\ WT}$) or *Xiap*$^{-/-}$ ($WT^{BL6\ XIAP-/-}$) hematopoietic cells. After 60 days, when the complete hematopoietic compartment was exchanged, mice were again subject to irradiation and transplantation, this time with bone marrow and T cells from allogenic Balb/c mice.

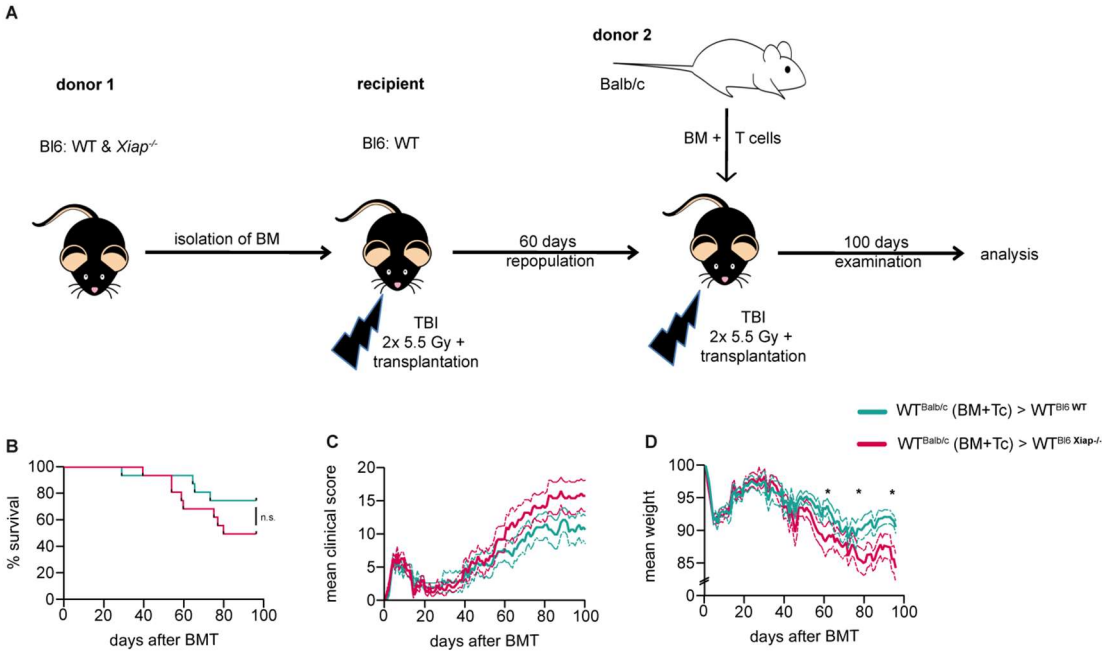


Figure 4. 29.: Increased GvHD incidence is dependent on XIAP loss in the hematopoietic compartment.

(A) Experimental setup for bone marrow chimeras. (B) Kaplan-Meier survival curve of $WT^{BL6\ WT}$ (n=9) and $WT^{BL6\ XIAP-/-}$ (n=8) mice after Balb/c transplantation. (C) Mean clinical score of $WT^{BL6\ WT}$ (n=9) and $WT^{BL6\ XIAP-/-}$ (n=8) mice after Balb/c transplantation. (D) Mean weight curve of $WT^{BL6\ WT}$ (n=9) and $WT^{BL6\ XIAP-/-}$ (n=8) mice after Balb/c transplantation.

This second transplantation resulted in GvHD induction in both $WT^{BL6\ WT}$ and $WT^{BL6\ XIAP-/-}$ mice but showed more Xiap loss related GvHD deaths in $WT^{BL6\ XIAP-/-}$ chimeras (Fig.4.29.B). The increased death rate also correlated with a higher mean clinical score (fig.4.29.C) and significantly increased weight loss in $WT^{BL6\ XIAP-/-}$ chimeras (Fig.4.29.D).

Summarized it can be said, that loss of XIAP increases GvHD induction and severity, based on a Th1 driven response, which is initiated by XIAP deficient hematopoietic cells.

5. Discussion

5.1. Loss of XIAP E3 ligase function triggers a cascade of inflammatory reactions

The family of IAP proteins was initially discovered for their ability to block caspases and thereby circumvent death (Clem, Fechheimer, and Miller 1991). Only later it was uncovered that the only true caspase inhibitor is XIAP and that other IAPs, like cIAP1/2 can prevent death via their role downstream of the TNF receptor. By modifying RIPK1 with ubiquitin chains, cIAP1/2 prevents death by retaining RIPK1 in complex I and therefor preventing engagement in complex II for apoptosis induction (Bertrand et al. 2008). Due to the fact that XIAP was associated only with caspase inhibition via XIAP´s BIR domains, XIAP E3 ligase function was neglected for quite some time. Within the last ten years, the Ring domain of XIAP and its function became the focus of several research fields. It was discovered that XIAP can modify, just as cIAP1/2, many interacting proteins by adding various ubiquitin chains. Examples for this control are XIAP self-regulation (Y. Yang et al. 2000) but also death unrelated signaling events including cell motility or NOD2 innate immune signaling (Damgaard et al. 2012).

In a recent, lab own publication, it was shown that loss of XIAP, or loss of its Ring functionality, and stimulation with TLR ligands resulted in inflammatory death of dendritic cells. To evaluate the consequences of this inflammatory cell death on the surrounding environment, a coculture system was developed. It could be shown that T cells cocultured with treated *Xiap*$^{-/-}$ BMDCs were specifically polarized to produce IL–17, but no IFNγ. This was true for the majority of innate like T cells, including $\gamma\delta$, DN T cells and iNKTs, all subsets known to be inducible without T cell receptor engagement and only via cytokines like IL–1β, IL–23 or a combination of both (Sutton et al. 2009; Doisne et al. 2011; Ueyama et al. 2017). Nonetheless it has to be stated, that the survival of innate like and CD4^{+} T lymphocytes was dependent on the dendritic cells, as the coculture with only cytokine enriched supernatant resulted in more

than 90% dead T cells and no induction of IL-17. Which may hint to a probable self-antigen presentation, or presentation of antigens from media and FCS. Moreover, a small fraction of $CD4^+$ cells also reacted with IL-17 production after coculture with *Xiap*$^{-/-}$ BMDCs. Sutton and colleagues described that IL-17 producing $\gamma\delta$ T cells were able to induce IL-17 production in $CD4^+$ cells in an amplification loop (Sutton et al. 2009). Although $CD4^+$ cells were not further specified, they might be innate memory-phenotype $CD4^+$ cells, a subset described to react to cytokines without TCR engagement (J. Hu and Avery August 2008; Takeshi Kawabe et al. 2017).

With the help of *Xiap*$^{\Delta Ring/\Delta Ring}$ BMDCs it was verified, that the induction of IL-17 was dependent on the E3 ligase activity of XIAP. Loss of the Ring domain functionality resulted in identical inflammatory cell death and a strong IL-1β induction and secretion, as seen after complete loss of XIAP. Experiments with recombinant IL-1β treatment and IL1R1 knockout T cells proved that innate like T cell subsets were exclusively dependent on IL-1β for their IL-17 induction, while $CD4^+$ cells were also dependent on IL-1β, but this cytokine alone was not sufficient to induce IL-17. This correlates with literature, stating that $CD4^+$ cells require the combination of IL-6 and TGF-β for a proper Th17 development (Bettelli et al. 2006; Veldhoen et al. 2006). Moreover, IL-23 has been shown to support maturation and thereby late differentiation (McGeachy et al. 2009), whereas IL-1 β facilitates expansion (Gulen et al. 2010) and is needed for early differentiation (Chung et al. 2009).

The implications for human patients of this *in vitro* data is underlined by a recent publication. Segura and colleagues were able to detect inflammatory dendritic cells from patients with chronic inflammatory diseases, capable of IL-17 induction (Segura et al. 2013). Furthermore, *in vivo* experiments with mice have shown a critical link between exaggerated IL-1β produced by dendritic cells leading to IL-17 induction in innate and adaptive T cell subsets and thereby promoting autoinflammatory reactions (SJ Lalor, J.Immunol 2011).

It can be concluded that loss of XIAP results in inflammatory death accompanied by secretion of IL-1β. This is followed by an activation of innate like and adaptive T lymphocytes as a result of the IL-1β signaling and

culminates in further inflammatory cytokine production, namely IL-17. These data, showing that aberrant IL-1β production can lead to highly inflammatory IL-17 induction, indicate that XLP-2 disease might at least in part be driven by hyperinflammatory responses downstream of IL-1β and IL-17.

5.2. Deductions from *in vitro* data for XLP-2 pathogenesis

XLP-2 symptoms can in summary be described as a hyperinflammatory overreaction of the immune system, with manifestations like HLH and IBD being the most prominent ones (Latour and Aguilar 2015; Aguilar and Latour 2015). Therefore, it can be hypothesized that the cause for this disease lies in the downstream effects of XIAP loss, which trigger inflammatory death which strongly drives further immune reactions and thereby starts a viscous cycle of inflammation.

Up to now the pathogenesis of XLP-2 disease is not understood at all. In 2015 S. Latour and C.Aguilar summarized the current knowledge, stating that the pathogenesis is probably based on three different aspects (Latour and Aguilar 2015). First, loss of XIAP results in a defective innate immune response with persistence of bacteria, as XIAP has been described to be necessary for NOD2 (Damgaard et al. 2012) and Dectin-1 signaling (Wan-Chen Hsieh et al. 2014).

Second, XIAP-deficiency triggers inflammatory death accompanied by IL-1β production (Yabal et al. 2014; James E Vince et al. 2012). This hyperinflammatory situation is exactly what is seen in the experimental coculture system, implicating, that results derived from this *in vitro* data, might well play a role in XLP-2 patients. Moreover, the additional induction of IL-17 as a proinflammatory cytokine might drive and enhance the inflammation induced by IL-1β. Interestingly, a publication from Taizo Wada reported significantly increased IL-18 in the serum of XLP-2 patients (Wada et al. 2014). Although this increase could not be allocated to spontaneous activation of PBMCs, this finding is of particular interest because of two reasons. IL-18 is,

like IL-1β, produced as a cytosolic precursor and needs processing by Caspase 1 in the inflammasome to be secreted as an active molecule (Gu et al. 1997). Moreover, there is evidence, that IL-18 may be able to substitute for IL-1β in the induction of IL-17 in innate T lymphocytes as well as in $CD4^+$ cells (Lalor et al. 2011). So, although the source of increased IL-18 in XLP-2 patients is unknown it might have downstream effects similar to what was seen in the experimental coculture setup.

The third aspect potentially involved in XLP-2 pathogenesis is a disrupted adaptive immune response by T cells, which leads to a persistence of virally infected cells. This assumption is based on observations that XIAP deficient T lymphocytes are more susceptible to activation-induced cell death after FAS, TRAIL-R or TCR stimulation (Rigaud et al. 2006; Marsh et al. 2010; Speckmann et al. 2013). These *in vitro* acquired data sets stand in contrast to reported symptoms. Especially hemophagocytic lymphohistiocytosis (HLH), which results from overwhelming macrophage and T lymphocyte activation, proliferation and accumulation, seems not to fit into this theory (Henter et al. 2007; Filipovich et al. 2010). Unfortunately, data concerning T lymphocytes in XLP-2 disease is not well defined. XLP-2 patients were initially included in XLP (SAP deficiency) patient groups, due to their closely related symptoms. With the discovery of XLP-2 being a distinct disease, many research groups investigated T lymphocytes, but did not report any aberrations in T cell counts, including $CD3^+$, $CD4^+$ and $CD8^+$ T cells (Rigaud et al. 2006; Speckmann et al. 2013). Specific focus however was given to iNKT cells by several groups, as this subset is absent in XLP-1 patients. Although no differences were reported for iNKT cell counts for XIAP deficient mice (Harlin et al. 2001; Rigaud et al. 2006; Bauler, Duckett, and O'Riordan 2008) several papers stated reduced numbers in XLP-2 patients (Rigaud et al. 2006; X. Yang, Miyawaki, and Kanegane 2012). In contrast, other papers state unaltered iNKT cell counts (Marsh et al. 2009; Speckmann et al. 2013). Conflicting data might well depend on the fact that iNKT cells are known for their capability to quickly downregulate TCR expression after TCR-mediated activation (Wilson et al. 2003), although a recent publication might explain reduced iNKT numbers by the discovery that PLZF induces a pro-apoptotic process that is counterbalanced by XIAP (Stephane Gerart et al. 2013). Up to now, no data

was published concerning γδ or double-negative T lymphocytes in XLP-2 patients, but following personal communication with H. Kanegane, no abnormalities in preliminary cell counts concerning these two subsets could be reported. This is of particular interest, because high PLZF expression was detected not only in iNKT cells, but also in γδ or DN T lymphocytes.

Concluding from the experimental data, it is likely, that XLP-2 pathogenesis is based not on three, but rather two aspects. Loss of XIAP results in deregulated innate signaling of Dectin-1 and NOD2 and secondly inflammatory death triggers a cascade of inflammatory cytokine induction as well as T cell activation and proliferation. Still, to understand the specific T cell subsets involved, their specific activation and their deregulation needs further evaluation.

5.3. RIPK3 at the crossroads of death and inflammation

Signaling downstream of death receptors like TNF, Fas or TRAIL has been of longstanding interest to researchers. With possibilities of survival, inflammation and various ways to die downstream of death receptors, several lines of research are crossing paths. At this point in time it seems like although so much knowledge is already gathered, still more questions arise then are solved. This is often due to the fact, that it is in most cases not clear which process comes first, with inflammation being a consequence of cell death or vice versa (Vince and Silke 2016).

RIPK3 and MLKL have been described as the two main critical regulators of necroptotic death independent of the activating stimulus. Through oligomerization and phosphorylation RIPK3 is activated (Li et al. 2012; Cho et al. 2009; He et al. 2009), recruits and activates MLKL through phosphorylation and thereby initiates necroptosis (Sun et al. 2012) (H. Wang et al. 2014). When experiments after XIAP loss showed a RIPK3-dependent induction of death and IL-1β processing, everything pointed towards necroptosis. In contrast to this, additional experiments could not verify an anticipated MLKL dependence.

MLKL and XIAP double deficient BMDCs showed an identical phenotype as *Xiap*$^{-/-}$ BMDCs, indicating no involvement of MLKL in death and IL–1β induction and therefore no role in IL–17 production. But at the same time Caspase 8 inhibition (Section 4.3.4) could not prevent death of BMDCs.

There might be two explanations why MLKL did not imitate the effects of RIPK3 loss. The first one suggests other effectors of RIPK3 being able to induce necroptosis. The concept of MLKL being the sole necroptosis inducing protein was already challenged one year ago by Zhang and his coworkers. They were capable to show that CAMKII, a calcium-dependent protein kinase, is also a target of RIPK3 phosphorylation. This modification leads to CAMKII activating ion channels in the plasma membrane and results in necroptosis (T. Zhang et al. 2016). So the concept of other RIPK3 targets with the ability to execute necroptosis is a valid option.

The second explanation is called the “switch theory”. The idea behind this theory is based on the concept that when death is triggered, it will be executed one way or another. Therefore, a cell who´s death fate is settled, will induce the signaling cascade available. One example for this was reported by Remijsen et al, where a switch from necroptosis to apoptosis occurred when RIPK3 or MLKL were depleted in L929 cells (Remijsen et al. 2014). In the same year Cook and his lab also stated that once RIPK3 is activated the mode of cell death was determined by the availability of downstream molecules (Cook et al. 2014). Moreover, the possibility of a switch between pathways was further reinforced only a year later. This third publication showed that in absence of IAPs, LPS triggered caspase 8 driven apoptosis independently of RIPK3 kinase or MLKL. When caspase 8 was additionally blocked an instant switch to necroptosis via RIPK3 and MLKL occurred (Lawlor et al. 2015). This situation is comparable to what is seen after loss of XIAP alone. Thus, XIAP might control induction of death in dependence of the available molecules, but with RIPK3 as a critical downstream effector. This would mean induction of apoptosis when MLKL is deleted and occurrence of necroptosis when Caspase 8 is blocked.

Moreover, RIPK1 kinase activity suppression did not change the loss of viability witnessed after TNF treatment (Section 4.4.1). Thus, it can be

concluded that the kinase function of RIPK1 is not needed for death induction after loss of XIAP. This is particular interesting, as so far RIPK1 kinase activity has been described as being essential for induction of necroptosis downstream of the TNF receptor (Holler et al. 2000), while being necessary for apoptosis under certain circumstances (L. Wang, Du, and Wang 2008; Dondelinger et al. 2013). Although one report states induction of necroptosis without RIPK1 is possible, this data is based on an artificial RIPK3 overexpression (Moujalled et al. 2013). Consequently, this data cannot be considered for the processes after loss of XIAP, especially because RIPK3 was shown not to be upregulated upon XIAP-deficiency (Yabal et al. 2014). Moreover, it was surprising to see that RIPK1 kinase activity was necessary for Caspase 1 dependent IL-1β processing, in comparison to Caspase 8 dependent processing (Section 4.4.1). Moreover Yabal et al. reported an increased ubiquitination status of RIPK1 after loss of XIAP (Yabal et al. 2014). Hence, the role of RIPK1 in XIAP dependent signaling will need further investigation.

With the idea of XIAP being able to regulate apoptosis as well as necroptosis in a RIPK3 dependent manner, a critical question arises. If after MLKL knockout a caspase 8 driven apoptotic death occurs, how can it be that features of programmed necrosis are still detectable? That, at least in some part, lytic cell death occurred was proven by LDH detection (Section 4.1.3), but this might well be derived from the minor fraction of pyroptosis proven by cleaved Gasdermin D (Section 4.3.4). This still does not explain the significant increase of proinflammatory IL-1β measured even after combined loss of XIAP and MLKL (Section 4.4.3). An explanation therefor might be found in recent discoveries. Within the last few years several publications revealed that RIPK1 and RIPK3 have several functions apart from necroptosis and have a broader range of responsibilities independent of necroptotic death, mostly with the involvement of inflammasome activation (Wong et al. 2014; Moriwaki et al. 2015) (Najjar et al. 2016). Of special interest is the work of Kim Newton, which showed, that MLKL was often not involved in autoinflammatory settings controlled by RIPK3 (K Newton et al. 2016).

The data from *Caspase1/11/Xiap*$^{-/-}$ BMDCs indicates that cell death and

inflammasome activation are two mostly separated processes. This is shown by the fact that cell death in double deficient mice or after Caspase 8 inhibition is comparable to XIAP deficient cells (except for the minor fraction of pyroptosis), while IL-1β processing is significantly dampened (Section 4.3.4). At this point it cannot be distinguished whether IL-1β secretion is an active process, or is passively released after membrane rupture. This leaves unresolved, whether inflammation induces cell death or inflammation is a consequence of cell death. Most likely this question is so hard to answer because RIPK3 can regulate both of these processes. Therefore, it can only be concluded, that in the context of XIAP deficiency, RIPK3 is the key molecule driving T cell polarization through inflammation.

5.4. Applicability from *in vitro* to *in vivo* situations

To translate acquired data from *in vitro* experiments to an animal model has always been a subject of discussion. The transfer is often flawed, as *in vitro* setups can never fully imitate the complicated system and represent therefore a simplified version of the in *vivo* situation.

Nevertheless, an attempt was made, to see whether *in vitro* coculture results could be translated into different *in vivo* settings to test the effect of XIAP loss. With the intraperitoneal LPS injection the XLP-2 patient situation was mimicked, where bacterial burdens have been described next to viral infections (Schmid et al. 2011). The results from this experiment correlated with the results obtained from coculture, with locally and systemically increased IL-1β and IL-17 leading to a neutrophil influx (Section 4.5.1). Although no abnormal neutrophil counts in the blood of XLP-2 patients were observed (Aguilar, Lenoir, et al. 2014), a localized recruitment of neutrophils through IL-17 in the gut may well play a role in IBD observed symptoms of XLP-2 patients. Neutrophil involvement in IBD is a debated topic, but the common perception is that in Crohn´s disease neutrophil function is impaired, while in Colitis neutrophils drive hyperinflammation (summarized by (Wéra, Lancellotti, and

Oury 2016)). While XLP-2 IBD symptoms are nowadays more associated with Crohn´s disease (Aguilar, MS, et al. 2014; Yvonne Zeissig et al. 2014), initial correlation was with Colitis (Rigaud et al. 2006; Schmid et al. 2011; Speckmann et al. 2013).

The concept that deregulated IL-1β levels contribute to disease pathology in XLP-2 patients might well be dependent on IL-17 in local tissues and not in a systemic manner. This could be important for instance in coherence with IBD symptoms detected in 25-30% of XLP-2 patients (Aguilar and Latour 2015), which can even occur before any other indication or be the only manifestation at all (Aguilar, MS, et al. 2014; Yvonne Zeissig et al. 2014). Up to now, IBD symptoms in XIAP deficient patients were associated by the defect in NOD2 signaling in the absence of XIAP (Damgaard et al. 2013). IBD disease progression might be further enhanced be deregulated inflammatory cell death disrupting the epithelial barrier and inflammation being fueled by IL-17, a cytokine closely connected to IBD pathogenesis (Catana et al. 2015). IBD-like symptoms can be mimicked by the Citrobacter rodentium mouse model (J. W. Collins et al. 2014). Results showed a heightened susceptibility to Citrobacter rodentium infection, with increased bacterial burden, weight loss and inflammation in histological section of the colon (Section 5.4.2). Analysis of the intraepithelial compartment showed a marked increase in $CD4^+$ and γδ T cells, which correlated with increased IL-22 expression but no IL-17 upregulation. This is of particular interest, as IL-22 is frequently produced by the same T cell subsets as IL-17. But IL-22 induces production of antimicrobial peptides and tissue repair factors and is therefore associated with a protective role in IBD (Kilian, Valentina, and Andrea 2017).

In the third animal model, it was shown that loss of XIAP in recipient cells, leads to a more severe progression of GvHD after transplantation and this effect could be attributed to the hematopoietic compartment (Section 4.5.4). Interestingly, also IBD in XLP-2 patients was linked to abnormalities in the hematopoietic compartment, as successful HSCT cured all symptoms of IBD (Aguilar, MS, et al. 2014; Ono et al. 2016). Similar to the Citrobacter rodentium mouse model, no increase in $IL\text{-}17^+$ T cells was detectable, but instead a

significant upregulation of IFNγ as well as high proliferation of disease driving donor T cells was registered.

In summary it seems, that IL-17 does not play a role in XIAP loss driven IBD or GvHD, but this cannot be fully excluded, as IL-17 may play a role in an even earlier time point then analyzed.

How important inflammatory death and its regulation of immune responses is in diseases, is shown by the accumulating evidence from publications. Upregulation of RIPK3 and associated necroptosis was reported in several cases of tissue injury and inflammation. Examples can be found in the retinal epithelium (Trichonas et al. 2010), ischemia reperfusion injury (Linkermann et al. 2013), as well as in children with IBD (Pierdomenico et al. 2013). Additionally, there is also proof that necroptosis and TNF dependent signaling can play a role in hyper inflammatory syndromes like SIRS (Duprez et al. 2011). A study from Takahashi et al. even showed that TNF-driven shock is mediated by IL-17, linking it to the *in vitro* results detected in this work (N. Takahashi et al. 2008). Another link between the presented *in vitro* data and the *in vivo* situation is a publication where it was shown that murine and human γδ T lymphocytes can be activated by dendritic cells dying in a necroptotic fashion (C. C. Collins et al. 2016).

All in all, increasing evidence stresses the importance of inflammatory death and is further supported by findings presented in this thesis. A thoroughly understanding of the underlying correlations will be a critical task for the next years to come for optimal therapeutic interventions in inflammatory death driven diseases and specifically XLP-2.

6. Bibliography

Aguilar, Claire, and Sylvain Latour. 2015. “X-Linked Inhibitor of Apoptosis Protein Deficiency: More Than an X-Linked Lymphoproliferative Syndrome..” *Journal of Clinical Immunology* 35 (4): 331–38. doi:10.1007/s10875-015-0141-9.

Aguilar, Claire, Christelle Lenoir MS, Nathalie Lambert AS, Bernadette Bègue MS, Nicole Brousse MD, Danielle Canioni MD, Dominique Berrebi MD PhD, et al. 2014. “Characterization of Crohn Disease in X-Linked Inhibitor of Apoptosis–Deficient Male Patients and Female Symptomatic Carriers.” *Journal of Allergy and Clinical Immunology* 134 (5). Elsevier Ltd: 1131–41. doi:10.1016/j.jaci.2014.04.031.

Aguilar, Claire, Christelle Lenoir, Nathalie Lambert, Bernadette Bègue, Nicole Brousse, Danielle Canioni, Dominique Berrebi, et al. 2014. “Characterization of Crohn Disease in X-Linked Inhibitor of Apoptosis-Deficient Male Patients and Female Symptomatic Carriers..” *The Journal of Allergy and Clinical Immunology* 134 (5): 1131–39. doi:10.1016/j.jaci.2014.04.031.

Alipour, Misagh, Yuefei Lou, Daniel Zimmerman, Michael W Bording-Jorgensen, Consolato Sergi, Julia J Liu, and Eytan Wine. 2013. “A Balanced IL-1β Activity Is Required for Host Response to Citrobacter Rodentium Infection.” Edited by Jörn Coers. *PLoS ONE* 8 (12): e80656. doi:10.1371/journal.pone.0080656.s005.

Andrew Kovalenko, Christine Chable-Bessia, Giuseppina Cantarella, Alain Israe l, and David Wallach Gilles Courtois. 2003. “The Tumour Suppressor CYLD Negatively Regulates NF-kB Signalling by Deubiquitination .” *Nature* 424 (August): 801–5. doi:10.1038/nature01811.

Antonelli, L R V, W O Dutra, R R Oliveira, K C L Torres, L H Guimaraes, O Bacellar, and K J Gollob. 2006. “Disparate Immunoregulatory Potentials for Double-Negative (CD4- CD8-) ?? and ?? T Cells From Human Patients with Cutaneous Leishmaniasis.” *Infection and Immunity* 74 (11): 6317–23. doi:10.1128/IAI.00890-06.

Bauler, Laura D, Colin S Duckett, and Mary X D O'Riordan. 2008. “XIAP Regulates Cytosol-Specific Innate Immunity to Listeria Infection.” Edited by Pascale Cossart. *PLoS Pathogens* 4 (8): 1–12. doi:10.1371/journal.ppat.1000142.s004.

Bendelac, Albert. 1995. “CD1:Presenting Unusual Antigens to Unusual T Lymphocytes.” *Science* 269 (July): 185–86.

Bendelac, Albert, Nigel Killeen, Dan R Littman, and Ronald H Schwartz. 1994. “A Subset of CD4+ Thymocytes Selected by MHC Class I Molecules.” *Science* 263 (March): 1774–77.

Bendelac, Albert, Paul B Savage, and Luc Teyton. 2007. “The Biology of NKT Cells.” *Annual Review of Immunology* 25 (1): 297–336. doi:10.1146/annurev.immunol.25.022106.141711.

Benedict, Chris A, Paula S Norria, and Carl F Ware. 2002. “To Kill or Be Killed: Viral Evasion of Apoptosis.” *Nature Immunology* 3 (11): 1013–18.

Berghe, Tom Vanden, Andreas Linkermann, Sandrine Jouan-Lanhouet, Henning Walczak, and Peter Vandenabeele. 2014. “Perspectives.” *Nature Publishing Group* 15 (2). Nature Publishing Group: 135–47.

doi:10.1038/nrm3737.
Berghe, Tom Vanden, Behrouz Hassannia, and Peter Vandenabeele. 2016. “An Outline of Necrosome Triggers.” *Cellular and Molecular Life Sciences* 73 (11). Springer International Publishing: 2137–52. doi:10.1007/s00018-016-2189-y.
Bertrand, Mathieu J M, Snezana Milutinovic, Kathleen M Dickson, Wai Chi Ho, Alain Boudreault, Jon Durkin, John W Gillard, James B Jaquith, Stephen J Morris, and Philip A Barker. 2008. “cIAP1 and cIAP2 Facilitate Cancer Cell Survival by Functioning as E3 Ligases That Promote RIP1 Ubiquitination.” *Molecular Cell* 30 (6): 689–700. doi:10.1016/j.molcel.2008.05.014.
Bettelli, Estelle, Yijun Carrier, Wenda Gao, Thomas Korn, Terry B Strom, Mohamed Oukka, Howard L Weiner, and Vijay K Kuchroo. 2006. “Reciprocal Developmental Pathways for the Generation of Pathogenic Effector TH17 and Regulatory T Cells..” *Nature* 441 (7090): 235–38. doi:10.1038/nature04753.
Beutler, Bruce A. 2009. “TLRs and Innate Immunity.” *Blood* 113 (7): 1399–1407. doi:10.1182/blood-2008.
Birnberg, Tal, Liat Bar-On, Anita Sapoznikov, Michele L Caton, Luisa Cervantes-Barragán, Divine Makia, Rita Krauthgamer, et al. 2008. “Lack of Conventional Dendritic Cells Is Compatible with Normal Development and T Cell Homeostasis, but Causes Myeloid Proliferative Syndrome.” *Immunity* 29 (6). Elsevier Inc.: 986–97. doi:10.1016/j.immuni.2008.10.012.
Bonneville, Marc, Rebecca L O'Brien, and Willi K Born. 2010. “T Cell Effector Functions: a Blendof Innate Programming and Acquiredplasticity.” *Nature Publishing Group* 10 (7). Nature Publishing Group: 467–78. doi:10.1038/nri2781.
Braun, Nicole A, Roman Covarrubias, and Amy S Major. 2010. “Natural Killer T Cells and Atherosclerosis: Form and Function Meet Pathogenesis.” *Journal of Innate Immunity* 2 (4): 316–24. doi:10.1159/000296915.
Brennan, Patrick J, Manfred Brigl, and Michael B Brenner. 2013. “Invariant Natural Killer T Cells:an Innate Activation Scheme Linkedto Diverse Effector Functions.” *Nature Reviews Immunology* 13 (2). Nature Publishing Group: 101–17. doi:10.1038/nri3369.
Brown, Gordon D. 2005. “Dectin-1: a Signalling Non-TLR Pattern-Recognition Receptor.” *Nature Reviews Immunology* 6 (1): 33–43. doi:10.4049/jimmunol.173.2.1284.
Budhidarmo, Rhesa, and Catherine L Day. 2015. “Seminars in Cell & Developmental Biology.” *Seminars in Cell and Developmental Biology* 39 (March). Elsevier Ltd: 80–90. doi:10.1016/j.semcdb.2014.12.002.
Carding, Simon R, and Paul J Egan. 2002. “Gamma Delta T CELLS: FUNCTIONAL PLASTICITY and HETEROGENEITY.” *Nature Reviews Immunology* 2 (5): 336–45. doi:10.1038/nri797.
Carl, Vogt. 1842. “Untersuchungen Über Die Entwicklungsgeschichte Der Geburtshelferkroete (Alytes Obstetricans).” *Solothurn: Jent & Gassmann*, July.
Catana, Cristina-Sorina, Ioana Berindan Neagoe, Vasile Cozma, Cristian Magdas, Flaviu Tabaran, and Dan Lucian Dumitrascu. 2015. “Contribution of the IL-17/IL-23 Axis to the Pathogenesis of Infammatory Bowel Disease.” *World Journal of Gastroenterology* 21 (19): 5823–30.

doi:10.3748/wjg.v21.i19.5823.
Chan, Francis Ka-Ming, Kenta Moriwaki, and María José De Rosa. 2013. “Detection of Necrosis by Release of Lactate Dehydrogenase Activity.” In *Methods in Molecular Biology*, 979:65–70. Methods in Molecular Biology. Totowa, NJ: Humana Press. doi:10.1007/978-1-62703-290-2_7.
Cheng, Min, and Shilian Hu. 2017. “Lung-Resident γδT Cells and Their Roles in Lung Diseases.” *Immunology* 10 (June): 467. doi:10.1111/j.1574-695X.2011.00840.x.
Cho, YoungSik, Sreerupa Challa, David Moquin, Ryan Genga, Tathagat Dutta Ray, Melissa Guildford, and Francis Ka-Ming Chan. 2009. “Phosphorylation-Driven Assembly of the RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation.” *Cell* 137 (6). Elsevier Inc.: 1112–23. doi:10.1016/j.cell.2009.05.037.
Chung, Yeonseok, Seon Hee Chang, Gustavo J Martinez, Xuexian O Yang, Roza Nurieva, Hong Soon Kang, Li Ma, et al. 2009. “Critical Regulation of Early Th17 Cell Differentiation by Interleukin-1 Signaling..” *Immunity* 30 (4): 576–87. doi:10.1016/j.immuni.2009.02.007.
Clem, Rollie J, Marcus Fechheimer, and Lois K Miller. 1991. “Prevention of Apoptosis by a Baculvirus Gene During Infection of Insect Cells ” *Science* 254 (November): 1388–90.
Collins, Cheryl C, Kathleen Bashant, Cuixia Erikson, Phyu Myat Thwe, Karen A Fortner, Hong Wang, Craig T Morita, and Ralph C Budd. 2016. “Necroptosis of Dendritic Cells Promotes Activation of Γδ T Cells.” *Journal of Innate Immunity* 8 (5): 479–92. doi:10.1159/000446498.
Collins, James W, Kristie M Keeney, Valerie F Crepin, Vijay A K Rathinam, Katherine A Fitzgerald, B Brett Finlay, and Gad Frankel. 2014. “Citrobacter Rodentium: Infection, in Ammation and the Microbiota.” *Nature Publishing Group* 12 (9). Nature Publishing Group: 612–23. doi:10.1038/nrmicro3315.
Constantinides, Michael G, and Albert Bendelac. 2013. “Trancriptional Regulation of the NKT Cell Lineage.” *Current Opinion in Immunology* 25 (2): 161–67.
Conti, Heather R, Alanna C Peterson, Lucas Brane, Anna R Huppler, Nydiaris Hern ndez-Santos, Natasha Whibley, Abhishek V Garg, et al. 2014. “Oral-Resident Natural Th17 Cells and ?? T Cells Control Opportunistic Candida Albicansinfections.” *Journal of Experimental Medicine* 211 (10): 2075–84. doi:10.1111/imr.12036.
Cook, W D, D M Moujalled, T J Ralph, P Lock, S N Young, J M Murphy, and D L Vaux. 2014. “RIPK1- and RIPK3-Induced Cell Death Mode Is Determined by Target Availability” 21 (10). Nature Publishing Group: 1600–1612. doi:10.1038/cdd.2014.70.
Corpuz, Theresa M, Jessica Stolp, Hee-Ok Kim, Gabriela V Pinget, Daniel H D Gray, Jae-Ho Cho, Jonathan Sprent, and Kylie E Webster. 2016. “Differential Responsiveness of Innate-Like IL-17– and IFN-Γ–Producing Γδ T Cells to Homeostatic Cytokines.” *The Journal of Immunology* 196 (2): 645–54. doi:10.4049/jimmunol.1502082.
Cowley, S C, A I Meierovics, J A Frelinger, Y Iwakura, and K L Elkins. 2010. “Lung CD4-CD8- Double-Negative T Cells Are Prominent Producers of IL-17A and IFN-? During Primary Respiratory Murine Infection with Francisella Tularensis Live Vaccine Strain.” *The Journal of Immunology* 184 (10): 5791–5801. doi:10.4049/jimmunol.1000362.

Crispin, Jose C, and George C Tsokos. 2009. “Human TCR- CD4- CD8- T Cells Can Derive From CD8 T Cells and Display an Inflammatory Effector Phenotype .” *Journal of Immunology (Baltimore, Md. : 1950)*. September 4. http://www.jimmunol.org/content/183/7/4675.

Crispin, Jose C, Mohammed Oukka, George Bayliss, Robert A Cohen, Christine A Van Beek, Isaac E Stillman, Vasileios C Kyttaris, Yuang-Taung Juang, and George C Tsokos. 2008. “Expanded Double Negative T Cells in Patients with Systemic Lupus Erythematosus Produce IL-17 and Infiltrate the Kidneys.” *Journal of Immunology (Baltimore, Md. : 1950)*, December, 1–14.

Crook, Norman E, Rollie J Clem, and Lois K Miller. 1993. “An Apoptosis-Inhibiting Baculovirus Gene with a Zinc Finger-LikeMotif.” *Journal of Virology* 67 (4): 2168–74.

Damgaard, Rune Busk, Berthe Katrine Fiil, Carsten Speckmann, Monica Yabal, Udo zur Stadt, Simon Bekker-Jensen, Philipp J Jost, Stephan Ehl, Niels Mailand, and Mads Gyrd-Hansen. 2013. “Disease-Causing Mutations in the XIAPBIR2 Domain Impair NOD2-Dependent Immune Signalling.” *EMBO Molecular Medicine* 5 (8): 1278–95. doi:10.1002/emmm.201303090.

Damgaard, Rune Busk, Ueli Nachbur, Monica Yabal, Wendy Wei-Lynn Wong, Berthe Katrine Fiil, Mischa Kastirr, Eva Rieser, et al. 2012. “The Ubiquitin Ligase XIAP Recruits LUBAC for NOD2 Signaling in Inflammationand Innate Immunity.” *Molecular Cell* 46 (6). Elsevier Inc.: 746–58. doi:10.1016/j.molcel.2012.04.014.

Deveraux, Quinn L, Ryosuke Takahashi, and Guy S Salvesen John C Reed. 1997. “X-Linked IAP Is a Directinhibitor of Cell-Death Proteases.” *Nature* 388 (July): 300–304.

Doisne, J M, V Soulard, C Becourt, L Amniai, P Henrot, C Havenar-Daughton, C Blanchet, et al. 2011. “Cutting Edge: Crucial Role of IL-1 and IL-23 in the Innate IL-17 Response of Peripheral Lymph Node NK1.1- Invariant NKT Cells to Bacteria.” *The Journal of Immunology* 186 (2): 662–66. doi:10.4049/jimmunol.1002725.

Dondelinger, Y, M A Aguileta, V Goossens, C Dubuisson, S Grootjans, E Dejardin, P Vandenabeele, and M J M Bertrand. 2013. “RIPK3 Contributes to TNFR1-Mediated RIPK1 Kinase-Dependent Apoptosis in Conditionsof cIAP1/2 Depletion or TAK1 Kinase Inhibition.” *Cell Death and Differentiation* 20 (10). Nature Publishing Group: 1381–92. doi:10.1038/cdd.2013.94.

Dondelinger, Yves, Maurice Darding, Mathieu J M Bertrand, and Henning Walczak. 2016. “Poly-Ubiquitination in TNFR1-Mediated Necroptosis.” *Cellular and Molecular Life Sciences* 73 (11). Springer International Publishing: 2165–76. doi:10.1007/s00018-016-2191-4.

Dong Zhang, Wei Yang, Nicolas Degauque, Yan Tian, Allison Mikita, and Xin Xiao Zheng. 2007. “New Differentiation Pathway for Double-Negative Regulatory T Cells That Regulates the Magnitude of Immune Responses.” *Blood*. May 1. http://www.bloodjournal.org/content/109/9/4071?sso-checked=true.

Du, Chunying, Min Fang, Yucheng Li, Lily Li, and Xiaodong Wang. 2000. “Smac, a Mitochondrial Protein That Promotes Cytochrome C–Dependent Caspase Activation by Eliminating IAP Inhibition,” June, 1–10.

Dunay, Ildiko R, Renato A DaMatta, Blima Fux, Rachel Presti, Suellen

Greco, Marco Colonna, and L David Sibley. 2008. “Gr1+ Inflammatory Monocytes Are Required for Mucosal Resistance to the Pathogen Toxoplasma Gondii.” *Immunity* 29 (2): 306–17. doi:10.1016/j.immuni.2008.05.019.

Duprez, Linde, Nozomi Takahashi, Filip Van Hauwermeiren, Benjamin Vandendriessche, Vera Goossens, Tom Vanden Berghe, Wim Declercq, Claude Libert, Anje Cauwels, and Peter Vandenabeele. 2011. “RIP Kinase-Dependent Necrosis Drives Lethal Systemic Inflammatory Response Syndrome.” *Immunity* 35 (6). Elsevier Inc.: 908–18. doi:10.1016/j.immuni.2011.09.020.

Duran-Struuck, Raimon, Adam Hartigan, Shawn G Clouthier, Melissa C Dyson, Kathi Lowler, Erin Gatza, Isao Tawara, et al. 2008. “Differential Susceptibility of C57BL/6NCr and B6.Cg-Ptprca Mice to Commensal Bacteria After Whole Body Irradiation in Translational Bone Marrow Transplant Studies.” *Journal of Translational Medicine* 6 (1): 10. doi:10.1186/1479-5876-6-10.

Eckelman, Brendan P, Guy S Salvesen, and Fiona L Scott. 2006. “Human Inhibitor of Apoptosis Proteins: Why XIAP Is the Black Sheep of the Family.” *EMBO Reports* 7 (10): 988–94. doi:10.1038/sj.embor.7400795.

Edinger, Matthias, Petra Hoffmann, Joerg Ermann, Kathryn Drago, C Garrison Fathman, Samuel Strober, and Robert S Negrin. 2003. “CD4+CD25+ Regulatory T Cells Preserve Graft-Versus-Tumor Activity While Inhibiting Graft-Versus-Host Disease After Bone Marrow Transplantation.” *Nature Medicine* 9 (9): 1144–50. doi:10.1038/nm915.

Emmanuel Martin, Emmanuel Treiner, Livine Duban, Lucia Guerri, He lene Laude, Ce cile Toly, Virginie Premel, et al. 2009. “*Stepwise Development of MAIT Cells in Mouse and Human*.” *PLoS Biology* 7 (3): 525–36. doi:10.1371/journal.

Engel, Isaac, Gr gory Seumois, Lukas Chavez, Daniela Samaniego-Castruita, Brandie White, Ashu Chawla, Dennis Mock, Pandurangan Vijayanand, and Mitchell Kronenberg. 2016. “Innate-Like Functions of Natural Killer T Cell Subsets Result From Highly Divergent Gene Programs.” *Nature Immunology* 17 (6): 728–39. doi:10.1186/s13059-014-0550-8.

Feng, Shanshan, Yonghui Yang, Yide Mei, Li Ma, De-e Zhu, Naseruddin Hoti, Mark Castanares, and Mian Wu. 2007. “Cleavage of RIP3 Inactivates Its Caspase-Independent Apoptosis Pathway by Removal of Kinase Domain.” *Cellular Signalling* 19 (10): 2056–67.

Ferrara, James LM, John E Levine, Pavan Reddy, and Ernst Holler. 2009. “Graft-Versus-Host Disease.” *The Lancet* 373 (9674): 1550–61. doi:10.1016/S0140-6736(09)60237-3.

Filipovich, A H, K Zhang, A L Snow, and R A Marsh. 2010. “X-Linked Lymphoproliferative Syndromes: Brothers or Distant Cousins?.” *Blood* 116 (18): 3398–3408. doi:10.1182/blood-2010-03-275909.

Fischer, K. 2005. “Isolation and Characterization of Human Antigen-Specific TCR??+ CD4-CD8- Double-Negative Regulatory T Cells.” *Blood* 105 (7): 2828–35. doi:10.1182/blood-2004-07-2583.

Franchi, Luigi, Neil Warner, Kyle Viani, and Gabriel Núñez. 2009. “Function of Nod-Like Receptors in Microbial Recognition and Host Defense.” *Immunological Reviews* 227 (1): 106–28. doi:10.4049/jimmunol.180.12.7859.

Fujii, Shin-ichiro, Kanako Shimizu, Caroline Smith, Laura Bonifaz, and Ralph M Steinman. 2003. “Activation of Natural Killer T Cells by A-Galactosylceramide Rapidly Induces the Full Maturation of Dendritic Cells in Vivo and Thereby Acts as an Adjuvant for Combined CD4 and CD8 T Cell Immunity to a Coadministered Protein.” *Journal of Experimental Medicine* 198 (2): 267–79. doi:10.1002/(SICI)1521-4141(199906)29:06<2014::AID-IMMU2014>3.0.CO;2-G.

Fulda, Simone, and Domagoj Vucic. 2012. “Targeting IAP Proteins for Therapeutic Intervention in Cancer.” *Nature Reviews Drug Discovery* 11 (2): 109–24. doi:10.1038/35046566.

Galli, Grazia, Paola Pittoni, Elena Tonti, Carmine Malzone, Yasushi Uematsu, Marco Tortoli, Domenico Maione, et al. 2007. “Invariant NKT Cells Sustain Specific B Cell Responses and Memory.” *Proceedings of the National Academy of Sciences of the United States of America* 104 (10): 3984–89.

Grishkan, Inna V, Achilles Ntranos, Peter A Calabresi, and Anne R Gocke. 2013. “Helper T Cells Down-Regulate CD4 Expression Upon Chronic Stimulation Giving Rise to Double-Negative T Cells.” *Cellular Immunology* 284 (1-2). Elsevier Inc.: 68–74. doi:10.1016/j.cellimm.2013.06.011.

Grootjans, Sasker, Tom Vanden Berghe, and Peter Vandenabeele. 2017. “Initiation and Execution Mechanisms of Necroptosis: an Overview” 24 (7). Nature Publishing Group: 1184–95. doi:10.1038/cdd.2017.65.

Gu, Yong, Keisuke Kuida, Hiroko Tsutsui, George Ku, Kathy Hsiao, Mark A Fleming, Nobuki Hayashi, et al. 1997. “Activation of Interferon-Gamma Inducing Factor Mediated by Interleukin-1 Converting Enzyme.” *Science* 275 (January): 206–9.

Gulen, Muhammet F, Zizhen Kang, Katarzyna Bulek, Wan Youzhong, Tae Whan Kim, Yi Chen, Cengiz Z Altuntas, et al. 2010. “The Receptor SIGIRR Suppresses Th17 Cell Proliferation via Inhibition of the Interleukin-1 Receptor Pathway and mTOR Kinase Activation..” *Immunity* 32 (1): 54–66. doi:10.1016/j.immuni.2009.12.003.

Hamad, Abdel Rahim A. 2010. “Analysis of Gene Profile, Steady State Proliferation and Apoptosis of Double-Negative T Cells in the Periphery and Gut Epithelium Provides New Insights Into the Biological Functions of the Fas Pathway.” *Immunologic Research* 47 (1-3): 134–42. doi:10.3109/08830189909088485.

Harlin, H, S B Reffey, C S Duckett, T Lindsten, and C B Thompson. 2001. “Characterization of XIAP-Deficient Mice.” *Molecular and Cellular Biology* 21 (10): 3604–8. doi:10.1128/MCB.21.10.3604-3608.2001.

Hägglöf, Thomas, Saikiran K Sedimbi, Jennifer L Yates, Roham Parsa, Briana Hauff Salas, Robert A Harris, Elizabeth A Leadbetter, and Mikael C I Karlsson. 2016. “Neutrophils License iNKT Cells to Regulate Self-Reactive Mouse B Cell Responses.” *Nature Immunology* 17 (12): 1407–14. doi:10.1002/eji.200939891.

He, Sudan, Lai Wang, Lin Miao, Tao Wang, Fenghe Du, Liping Zhao, and Xiaodong Wang. 2009. “Receptor Interacting Protein Kinase-3 Determines Cellular Necrotic Response to TNF-A.” *Cell* 137 (6). Elsevier Inc.: 1100–1111. doi:10.1016/j.cell.2009.05.021.

He, Sudan, Yuqiong Liang, Feng Shao, and Xiaodong Wang. 2011. “Toll-Like Receptors Activate Programmed Necrosis in Macrophages Through a

Receptor-Interacting Kinase-3-Mediated Pathway..” *Proceedings of the National Academy of Sciences of the United States of America* 108 (50): 20054–59. doi:10.1073/pnas.1116302108.

Heilig, Joseph S, and Susumu Tonegawa. 1986. “Diversity of Murine Γ Genes and Expression in Fetal and Adult T Lymphocytes.” *Nature* 332 (August): 836–40.

Helft, Julie, Jan Böttcher, Probir Chakravarty, Santiago Zelenay, Jatta Huotari, Barbara U Schraml, Delphine Goubau, and Caetano Reis e Sousa. 2015. “GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c.” *Immunity* 42 (6). Elsevier Inc.: 1197–1211. doi:10.1016/j.immuni.2015.05.018.

Henter, Jan-Inge, AnnaCarin Horne, Maurizio Aricó, R Maarten Egeler, Alexandra H Filipovich, Shinsaku Imashuku, Stephan Ladisch, et al. 2007. “HLH-2004: Diagnostic and Therapeutic Guidelines for Hemophagocytic Lymphohistiocytosis.” *Pediatric Blood & Cancer* 48 (2): 124–31. doi:10.1002/pbc.21039.

Hill, Geoffrey R, and James L M Ferrara. 2000. “The Primacy of the Gastrointestinal Tract as a Target Organ of Acute Graft-Versus-Host Disease: Rationale for the Use of Cytokine Shields in Allogeneic Bone Marrow Transplantation.” *Blood* 95 (9): 2754–59.

Holler, Nils, Rossana Zaru, Olivier Micheau, Margot Thome, Antoine Attinger, Salvatore Valitutti, Jean-Luc Bodmer, Pascal Schneider, Brian Seed, and Jürg Tschopp. 2000. “Fas Triggers an Alternative, Caspase-8−Independent Cell Death Pathway Using the Kinase RIP as Effector Molecule - Nature Immunology.” *Nature Immunology* 1 (6): 489–95. doi:10.1038/82732.

Hsu, Hailing, Hong-Bing Shu, Ming-Gui Pan, and David V Goeddel. 1996. “TRADD–TRAF2 and TRADD–FADD Interactions Define Two Distinct TNF Receptor 1Signal Transduction Pathways.” *Cell* 84 (January): 299–308.

Hu, Jianfang, and Avery August. 2008. “Naïve and Innate Memory Phenotype CD4+ T-Cells Have Different Requirements for Active Itk for Their Development.” *Journal of Immunology (Baltimore, Md. : 1950)* 180 (10): 6544–52.

Ivanov, Ivaylo I, Brent S McKenzie, Liang Zhou, Carlos E Tadokoro, Alice Lepelley, Juan J Lafaille, Daniel J Cua, and Dan R Littman. 2006. “The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells.” *Cell* 126 (6): 1121–33. doi:10.1016/j.cell.2006.07.035.

Iwasaki, A, and R Medzhitov. 2010. “Regulation of Adaptive Immunity by the Innate Immune System.” *Science* 327 (5963): 291–95. doi:10.1126/science.1183021.

James E Vince, W Wei-Lynn Wong, Ian Gentle, Kate E Lawlor, Ramanjaneyulu Allam, Lorraine OReilly, Kylie Mason, et al. 2012. “Inhibitor of Apoptosis Proteins Limit RIP3 Kinase-Dependent Interleukin-1 Activation .” *Immunity*. February 24. http://www.cell.com/immunity/abstract/S1074-7613(12)00050-7?_returnURL=http%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS1074761312000507%3Fshowall%3Dtrue.

Jankovic, Dragana, Jayanthi Ganesan, Michael Bscheider, Natalie Stickel, Felix C Weber, Greta Guarda, Marie Follo, et al. 2013. “The Nlrp3

Inflammasome Regulates Acute Graft-Versus-Host Disease." *Journal of Experimental Medicine* 210 (10): 1899–1910. doi:10.1182/blood-2007-06-094482.

Johansson, M, and N Lycke. 2003. "A Unique Population of Extrathymically Derived αβTCR+CD4−CD8− T Cells with Regulatory Functions Dominates the Mouse Female Genital Tract." *The Journal of Immunology* 170 (4): 1659–66. doi:10.4049/jimmunol.170.4.1659.

K NeWTon, D L Dugger, A Maltzman, J M Greve, M Hedehus, B Martin-McNulty, RAD Carano, et al. 2016. "RIPK3 Deficiency or Catalytically Inactive RIPK1 Provides Greater Benefit Than MLKL Deficiency in Mouse Models of Inflammation and Tissue Injury
" *Cell Death and Differentiation*. April 13. http://www.nature.com/cdd/journal/v23/n9/full/cdd201646a.html.

Kaczmarek, Agnieszka, Peter Vandenabeele, and Dmitri V Krysko. 2013. "Necroptosis: the Release of Damage-Associated Molecular Patterns and Its Physiological Relevance." *Immunity* 38 (2). Elsevier Inc.: 209–23. doi:10.1016/j.immuni.2013.02.003.

Kadena, T, G MATSUZAKI, S FUJISE, K KISHIHARA, H TAKIMOTO, M SASAKI, M BEPPU, S NAKAMURA, and t S NAKAMURAt K NOMOTO M SASAKIt M BEPPU. 2005. "TCRa/B CD4-CD8-T Cells Differentiate Extrathymically in an $_{\text{Ick-Independent}}$ Manner and Participate in Early Response Against Listeria Monocytogenes Infection Through Interferon-Y Production," December, 1–9.

Kaiser, William J, Haripriya Sridharan, Chunzi Huang, Pratyusha Mandal, Jason W Upton, Peter J Gough, Clark A Sehon, Robert W Marquis, John Bertin, and Edward S Mocarski. 2013. "Toll-Like Receptor 3-Mediated Necrosis via TRIF, RIP3, and MLKL." *The Journal of Biological Chemistry* 288 (43): 31268–79.

Kayagaki, Nobuhiko, Irma B Stowe, Bettina L Lee, Karen O'Rourke, Keith Anderson, Søren Warming, Trinna Cuellar, et al. 2015. "Caspase-11 Cleaves Gasdermin D for Non-Canonical Inflammasome Signalling." *Nature* 526 (7575): 666–71. doi:10.1038/nature15541.

Kilian, Eyerich, Dimartino Valentina, and Cavani Andrea. 2017. "IL-17 and IL-22 in Immunity: Driving Protection and Pathology." *European Journal of Immunology* 47 (4): 607–14.

Kim, Ji Hyung, Hye Sung Kim, Hye Young Kim, Sae Jin Oh, and Doo Hyun Chung. 2012. "Direct Engagement of TLR4 in Invariant NKT Cells Regulates Immune Diseases by Differential IL-4 and IFN-Γ Production in Mice." Edited by Geetha P Bansal. *PLoS ONE* 7 (9): e45348. doi:10.1371/journal.pone.0045348.s001.

Kitamura, Hidemitsu, Kenji Iwakabe, TakashiYahata, Shin-ichiro Nishimura, Akio Ohta, Yasushi Ohmi, Marimo Sato, et al. 1999. "The Natural Killer T (NKT) Cell Ligand -Galactosylceramide Demonstrates Its Immunopotentiating Effect by Inducing Interleukin (IL)-12 Production by Dendritic Cells and IL-12 Receptor Expression on NKT Cells ." *Journal of Experimental Medicine* 189 (7): 1121–27.

Kohlgruber, Ayano C, Carlos A Donado, Nelson M LaMarche, Michael B Brenner, and Patrick J Brennan. 2016. "Activation Strategies for Invariant Natural Killer T Cells." *Immunogenetics*, August. Immunogenetics, 1–15. doi:10.1007/s00251-016-0944-8.

Kovalovsky, Damian, Olisambu U Uche, Sonia Eladad, Robin M Hobbs,

Woelsung Yi, Eric Alonzo, Kevin Chua, et al. 2008. “The BTB–Zinc Finger Transcriptional Regulator PLZF Controls the Development of Invariant Natural Killer T Cell Effector Functions.” *Nature Immunology* 9 (9): 1055–64. doi:10.1038/ni.1641.

Kreslavsky, Taras, Adam K Savage, Robin Hobbs, Fotini Gounari, Roderick Bronson, Pablo Pereira, Pier Paolo Pandolfi, Albert Bendelac, Harald von Boehmer. 2009. “TCR-Inducible PLZF Transcription Factor Required for Innate Phenotype of a Subset of T Cells with Restricted TCR Diversity.” *Proceedings of the National Academy of Sciences of the United States of America* 106 (30): 12453–58.

Krysko, Dmitri V, Tom Vanden Berghe, Katharina D’Herde, and Peter Vandenabeele. 2008. “Apoptosis and Necrosis: Detection, Discrimination and Phagocytosis.” *Methods* 44 (3): 205–21. doi:10.1016/j.ymeth.2007.12.001.

Kumar, H, A Belperron, S W Barthold, and L K Bockenstedt. 2000. “Cutting Edge: CD1d Deficiency Impairs Murine Host Defense Against the Spirochete, Borrelia Burgdorferi.” *The Journal of Immunology* 165 (9): 4797–4801. doi:10.4049/jimmunol.165.9.4797.

Lalor, S J, L S Dungan, C E Sutton, S A Basdeo, J M Fletcher, and K H G Mills. 2011. “Caspase-1-Processed Cytokines IL-1beta and IL-18 Promote IL-17 Production by Γδ and CD4 T Cells That Mediate Autoimmunity.” *The Journal of Immunology* 186 (10): 5738–48. doi:10.4049/jimmunol.1003597.

Lanier, Lewis L, Ruitenberg Joyce J, and Phillips Joseph H. 1986. “Human CD3+ T Lymphocytes That Express Neither CD4 nor CD8 Antigens” 164 (July): 339–44.

LAROCCO, MARK T, and SUSAN J BURGERT. 1997. “Infection in the Bone Marrow Transplant Recipient and Role of the Microbiology Laboratory in Clinical Transplantation.” *Clinical Microbiology Reviews* 10 (2): 277–97.

Latour, Sylvain. 2007. “Natural Killer T Cells and X-Linked Lymphoproliferative Syndrome.” *Curr Opin Allergy Clin Immunol*, no. 7 (November): 510–14.

Latour, Sylvain, and Claire Aguilar. 2015. “XIAP Deficiency Syndrome in Humans..” *Seminars in Cell and Developmental Biology* 39 (March): 115–23. doi:10.1016/j.semcdb.2015.01.015.

Lawlor, Kate E, Nufail Khan, Alison Mildenhall, Motti Gerlic, Ben A Croker, Akshay A D rsquo Cruz, Cathrine Hall, et al. 2015. “RIPK3 Promotes Cell Death and NLRP3 Inflammasome Activation in the Absence of MLKL.” *Nature Communications* 6 (February). Nature Publishing Group: 1–19. doi:10.1038/ncomms7282.

Lee, Chih-Hung, Shi-Bei Wu, Chien-Hui Hong, Hsin-Su Yu, and Yau-Huei Wei. 2013. “Molecular Mechanisms of UV-Induced Apoptosis and Its Effects on Skin Residential Cells: the Implication in UV-Based Phototherapy.” *International Journal of Molecular Sciences* 14 (3): 6414–35. doi:10.3390/ijms14036414.

Li, Jixi, Thomas McQuade, Ansgar B Siemer, Johanna Napetschnig, Kenta Moriwaki, Yu-Shan Hsiao, Ermelinda Damko, et al. 2012. “The RIP1/RIP3 Necrosome Forms a Functional Amyloid Signaling Complex Required for Programmed Necrosis.” *Cell* 150 (2): 339–50. doi:10.1016/j.cell.2012.06.019.

Lin, Yong, Anne Devin, Yolanda Rodriguez, and Zheng-gang Liu. 1999.

"Cleavage of the Death Domain Kinase RIP by Caspase-8 Prompts TNF-Induced Apoptosis." *Genes & Development* 13 (August): 2514–26.

Linkermann, Andreas, Jan H Bräsen, Nina Himmerkus, Shuya Liu, Tobias B Huber, Ulrich Kunzendorf, and Stefan Krautwald. 2012. "Rip1 (Receptor-Interacting Protein Kinase 1)Mediates Necroptosis and Contributes to Renalischemia/Reperfusion Injury." *Kidney International* 81 (8). Elsevier Masson SAS: 751–61. doi:10.1038/ki.2011.450.

Linkermann, Andreas, Jan Hinrich Brasenb, Maurice Darding, Mi Kyung Jin, Ana B Sanz, Jan-Ole Heller, Federica De Zen, et al. 2013. "Two Independent Pathways of Regulated Necrosis Mediate Ischemia–Reperfusion Injury ." *Proceedings of the National Academy of Sciences of the United States of America* 110 (29): 12024–29. doi:10.1073/pnas.1305538110/-/DCSupplemental.

Liu, Xing, Zhibin Zhang, Jianbin Ruan, Youdong Pan, Venkat Giri Magupalli, Hao Wu, and Judy Lieberman. 2016. "Inflammasome-Activated Gasdermin D Causes Pyroptosis by Forming Membrane Pores." *Nature* 535 (7610). Nature Publishing Group: 153–58. doi:10.1038/nature18629.

Lockhart, E, A M Green, and J L Flynn. 2006. "IL-17 Production Is Dominated by ?? T Cells Rather Than CD4 T Cells During Mycobacterium Tuberculosis Infection." *The Journal of Immunology* 177 (7): 4662–69. doi:10.4049/jimmunol.177.7.4662.

Mace, Peter D, Callum Smits, David L Vaux, John Silke, and Catherine L Day. 2010. "Asymmetric Recruitment of cIAPs by TRAF2." *Journal of Molecular Biology* 400 (1). Elsevier Ltd: 8–15. doi:10.1016/j.jmb.2010.04.055.

Makino, Yasuhiko, Rieko Kanno, Toshihiro Ito, Kazuko Higashino, and Masaru Taniguchi. 1995. "Predominant Expression of Invariant V_{Alpha}14+ TCR a Chain in NK1.1+ T Cell Populations ." *International Immunology* 7 (7): 1157–61.

Marsh, R A, K Rao, P Satwani, K Lehmberg, I Muller, D Li, M O Kim, et al. 2013. "Allogeneic Hematopoietic Cell Transplantation for XIAP Deficiency: an International Survey Reveals Poor Outcomes." *Blood* 121 (6): 877–83. doi:10.1182/blood-2012-06-432500.

Marsh, R A, L Madden, B J Kitchen, R Mody, B McClimon, M B Jordan, J J Bleesing, K Zhang, and A H Filipovich. 2010. "XIAP Deficiency: a Unique Primary Immunodeficiency Best Classified as X-Linked Familial Hemophagocytic Lymphohistiocytosis and Not as X-Linked Lymphoproliferative Disease." *Blood* 116 (7): 1079–82. doi:10.1182/blood-2010-01-256099.

Marsh, Rebecca A, Joyce Villanueva, Mi-Ok Kim, Kejian Zhang, Dan Marmer, Kimberly A Risma, Michael B Jordan, Jack J Bleesing, and Alexandra H Filipovich. 2009. "Patients with X-Linked Lymphoproliferative Disease Due to BIRC4 Mutation Have Normal Invariant Natural Killer T-Cell Populations." *Clinical Immunology* 132 (1): 116–23. doi:10.1016/j.clim.2009.03.517.

Martin, Bruno, Keiji Hirota, Daniel J Cua, Brigitta Stockinger, and Marc Veldhoen. 2009. "Interleukin-17-Producing Γδ T Cells Selectively Expand in Response to Pathogen Products and Environmental Signals." *Immunity* 31 (2): 321–30. doi:10.1016/j.immuni.2009.06.020.

Martin, Seamus J. 2016. "Cell Death and Inflammation: the Case for IL-1 Family Cytokines as the Canonical DAMPs of the Immune System."

FEBS Journal 283 (14): 2599–2615. doi:10.1111/febs.13775.
Martina, Maria N, Sanjeev Noel, Ankit Saxena, Hamid Rabb, and Abdel Rahim A Hamad. 2015. “Double Negative (DN) Aβ T Cells: Misperception and Overdue Recognition..” *Immunology and Cell Biology* 93 (3): 305–10. doi:10.1038/icb.2014.99.
Matzinger, Polly. 1994. “Tolerance, Danger and the Extended Family.” *Annual Review of Immunology* 12 (August): 991–1045.
McGeachy, Mandy J, Yi Chen, Cristina M Tato, Arian Laurence, Barbara Joyce-Shaikh, Wendy M Blumenschein, Terrill K McClanahan, John J O'Shea, and Daniel J Cua. 2009. “The Interleukin 23 Receptor Is Essential for the Terminal Differentiation of Interleukin 17–Producing Effector T Helper Cells in Vivo.” *Nature Immunology* 10 (3): 314–24. doi:10.1038/ni.1698.
Merad, Miriam, Priyanka Sathe, Julie Helft, Jennifer Miller, and Arthur Mortha. 2013. “The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting.” *Annual Review of Immunology* 31 (1): 563–604. doi:10.1146/annurev-immunol-020711-074950.
Michel, Marie-Laure, Alexandre Castro Keller, Christophe Paget, Masakazu Fujio, François Trottein, Paul B Savage, Chi-Huey Wong, Elke Schneider, Michel Dy, and Maria C Leite-de-Moraes. 2007. “Identification of an IL-17–Producing NK1.1 negiNKT Cell Population Involved in Airway Neutrophilia.” *Journal of Experimental Medicine* 204 (5): 995–1001. doi:10.1002/eji.200535268.
Michel, Marie-Laure, Daniella Mendes-da-Cruz, Alexandre Castro Kellera, Matthias Lochner, Elke Schneider, Michel Dy, Gerard Eberl, Maria C Leite-de-Moraes. 2008. “Critical Role of ROR-Gt in a New Thymic Pathway Leading to IL-17-Producing Invariant NKT Cell Differentiation.” *Proceedings of the National Academy of Sciences of the United States of America* 105 (50): 19845–50.
Mildner, Alexander, and Steffen Jung. 2014. “Development and Function of Dendritic Cell Subsets.” *Immunity* 40 (5). Elsevier Inc.: 642–56. doi:10.1016/j.immuni.2014.04.016.
Misiak, Alicja, Mieszko M Wilk, Mathilde Raverdeau, and Kingston H G Mills. 2016. “IL-17?Producing Innate and Pathogen-Specific Tissue Resident Memory ?? T Cells Expand in the Lungs of Bordetella Pertussis?Infected Mice.” *The Journal of Immunology* 198 (1): 363–74. doi:10.4049/jimmunol.1601024.
Mixter, Philip F, Jennifer Q Russell, Gregory J Morrissette, Colette Charland, Deborah Aleman-Hoey, and Ralph C Budd. 1999. “A Model for the Origin of TCR- CD4 CD8 B220 Cells Based on High Affinity TCR Signals” 162 (May): 5747–56.
Moriwaki, Kenta, John Bertin, Peter J Gough, and Francis Ka-Ming Chan. 2015. “A RIPK3–Caspase 8 Complex Mediates Atypical Pro–IL-1β Processing.” *The Journal of Immunology* 194 (4): 1938–44. doi:10.4049/jimmunol.1402167.
Moujalled, D M, W D Cook, T Okamoto, J Murphy, K E Lawlor, J E Vince, and D L Vaux. 2013. “TNF Can Activate RIPK3 and Cause Programmed Necrosis in the Absence of RIPK1.” *Cell Death and Disease* 4 (1). Nature Publishing Group: e465–68. doi:10.1038/cddis.2012.201.
Muñoz-Ruiz, Miguel, Nital Sumaria, Daniel J Pennington, and Bruno Silva-

Santos. 2017. “Thymic Determinants of Gd T Cell Differentiation.” *Trends in Immunology* 38 (5). Elsevier Ltd: 336–44. doi:10.1016/j.it.2017.01.007.
Murphy, Kenneth, Paul Travers, and Mark Walport. 2008. *Janeway´S Immuno Biology*. 7 ed. Garland Science.
Najjar, Malek, Danish Saleh, Matija Zelic, Shoko Nogusa, Saumil Shah, Albert Tai, Joshua N Finger, et al. 2016. “RIPK1 and RIPK3 Kinases Promote Cell-Death- Independent Inflammation by Toll-Like Receptor 4.” *Immunity* 45 (1). Elsevier Inc.: 46–59. doi:10.1016/j.immuni.2016.06.007.
Nakatani, Yoshio, Torsten Kleffmann, Katrin Linke, Stephen M Condon, Mark G Hinds, and Catherine L Day. 2013. “Regulation of Ubiquitin Transfer by XIAP, a Dimeric RING E3 Ligase .” *Biochemical Journal* 450 (3): 629–38. doi:10.1073/pnas.93.25.14440.
Narayan, Kavitha, Katelyn E Sylvia, Nidhi Malhotra, Catherine C Yin, Gregory Martens, Therese Vallerskog, Hardy Kornfeld, et al. 2012. “Intrathymic Programming of Effector Fates in Three Molecularly Distinct ?? T Cell Subtypes.” *Nature Immunology* 13 (5): 511–18. doi:10.1016/S1534-5807(01)00003-X.
Nembrini, C, B Abel, M Kopf, and B J Marsland. 2006. “Strong TCR Signaling, TLR Ligands, and Cytokine Redundancies Ensure Robust Development of Type 1 Effector T Cells.” *The Journal of Immunology* 176 (12): 7180–88. doi:10.4049/jimmunol.176.12.7180.
NIEUWENHUIS, EDWARD E S, TETSUYA MATSUMOTO, MARK EXLEY, ROBBERT A SCHLEIPMAN, JONATHAN GLICKMAN, DAN T BAILEY, NADIA CORAZZA, SEAN P COLGAN, and ANDREW B ONDERDONK RICHARD S BLUMBERG. 2002. “CD1d-Dependent Macrophage-Mediated Clearance of Pseudomonas Aeruginosa From Lung.” *Nature Medicine* 8 (6): 588–93.
O Brien, Rebecca L, and Willi K Born. 2010. “Γδ T Cell Subsets: a Link Between TCR and Function?.” *Seminars in Immunology* 22 (4): 193–98. doi:10.1016/j.smim.2010.03.006.
Oberoi, Tripat Kaur, Taner Dogan, Jennifer C Hocking, Rolf-Peter Scholz, Juliane Mooz, Carrie L Anderson, Christiaan Karreman, et al. 2011. “IAPs Regulate the Plasticity of Cell Migration by Directly Targeting Rac1 for Degradation.” *The EMBO Journal* 31 (1). Nature Publishing Group: 14–28. doi:10.1038/emboj.2011.423.
Oberst, Andrew. 2015. “Death in the Fast Lane: What's Next for Necroptosis?.” *FEBS Journal* 283 (14): 2616–25. doi:10.1038/cdd.2015.70.
Ofengeim, Dimitry, and Junying Yuan. 2013. “Regulation of RIP1 Kinase Signalling at the Crossroads of Inflammation and Cell Death.” *Nat Rev Molecular Cell Biology* 14 (11). Nature Publishing Group: 727–36. doi:10.1038/nrm3683.
Ofengeim, Dimitry, Yasushi Ito, Ayaz Najafov, Yaoyang Zhang, Bing Shan, Judy Park DeWitt, Juanying Ye, et al. 2015. “Activation of Necroptosis in Multiple Sclerosis.” *Cell Reports* 10 (11): 1836–49. doi:10.1016/j.celrep.2015.02.051.
Ono, Shintaro, Tsubasa Okano, Akihiro Hoshino, Masakatsu Yanagimachi, Kazuko Hamamoto, Yozo Nakazawa, Toshihiko Imamura, et al. 2016. “Hematopoietic Stem Cell Transplantation for XIAP Deficiency in Japan.” *Journal of Clinical Immunology*, December. Journal of Clinical Immunology, 1–7. doi:10.1007/s10875-016-0348-4.

Paget, C, S Ivanov, J Fontaine, F Blanc, M Pichavant, J Renneson, E Bialecki, et al. 2011. “Potential Role of Invariant NKT Cells in the Control of Pulmonary Inflammation and CD8+ T Cell Response During Acute Influenza a Virus H3N2 Pneumonia.” *The Journal of Immunology* 186 (10): 5590–5602. doi:10.4049/jimmunol.1002348.

Papotto, Pedro H, Julie C Ribot, and Bruno Silva-Santos. 2017. “IL-17+ Γδ T Cells as Kick-Starters of Inflammation .” *Nature Immunology* 18 (6): 604–11. doi:10.1038/ncomms8464.

Park, Sun-Mi, Jong-Bok Yoon, and Tae H Lee. 2004. “Receptor Interacting Protein Is Ubiquitinated by Cellular Inhibitor of Apoptosis Proteins (C-IAP1 and C-IAP2) in Vitro.” *FEBS Letters* 566 (1-3): 151–56. doi:10.1074/jbc.M001202200.

Passos, Sara T, Jonathan S Silver, Aisling C O’Hara, David Sehy, Jason S Stumhofer, and Christopher A Hunter. 2010. “IL-6 Promotes NK Cell Production of IL-17 During Toxoplasmosis.” *The Journal of Immunology* 184 (4): 1776–83. doi:10.4049/jimmunol.0901843.

Pellicci, Daniel G, Kirsten J L Hammond, Adam P Uldrich, Alan G Baxter, Mark J Smyth, and Dale I Godfrey. 2002. “A Natural Killer T (NKT) Cell Developmental Pathway Involving a Thymus-Dependent NK1.1 CD4 CD1d-Dependent Precursor Stage.” *Journal of Experimental Medicine* 195 (7): 835–44.

Peternel, S, and M Kaštelan. 2009. “Immunopathogenesis of Psoriasis: Focus on Natural Killer T Cells.” *Journal of the European Academy of Dermatology and Venereology* 23 (10): 1123–27. doi:10.4049/jimmunol.180.8.5167.

Pichavant, Muriel, Sho Goya, Everett H Meyer, Richard A Johnston, Hye Y Kim, Ponpan Matangkasombut, Ming Zhu, et al. 2008. “Ozone Exposure in a Mouse Model Induces Airway Hyperreactivity That Requires the Presence of Natural Killer T Cells and IL-17.” *Journal of Experimental Medicine* 205 (2): 385–93. doi:10.1152/japplphysiol.00381.2001.

Pierdomenico, Maria, Anna Negroni, Laura Stronati, Roberta Vitali, Enrica Prete, John Bertin, Peter J Gough, Marina Aloi, and Salvatore Cucchiara. 2013. “Necroptosis Is Active in Children with Inflammatory Bowel Disease and Contributes to Heighten Intestinal Inflammation.” *The American Journal of Gastroenterology* 109 (2). Nature Publishing Group: 279–87. doi:10.1038/ajg.2013.403.

Priatel, J J, O Utting, and H S Teh. 2001. “TCR/Self-Antigen Interactions Drive Double-Negative T Cell Peripheral Expansion and Differentiation Into Suppressor Cells.” *The Journal of Immunology* 167 (11): 6188–94. doi:10.4049/jimmunol.167.11.6188.

Ranson, T, S Bregenholt, A Lehuen, O Gaillot, M C Leite-de-Moraes, A Herbelin, P Berche, and J P Di Santo. 2005. “Invariant v?14+ NKT Cells Participate in the Early Response to Enteric Listeria Monocytogenes Infection.” *The Journal of Immunology* 175 (2): 1137–44. doi:10.4049/jimmunol.175.2.1137.

Remijsen, Q, V Goossens, S Grootjans, C Van den Haute, N Vanlangenakker, Y Dondelinger, R Roelandt, et al. 2014. “Depletion of RIPK3 or MLKL Blocks TNF-Driven Necroptosis and Switches Towards a Delayed RIPK1 Kinase-Dependent Apoptosis” 5 (1). Nature Publishing Group: e1004–8. doi:10.1038/cddis.2013.531.

Rigaud, Stéphanie, Marie-Claude Fondanèche, Nathalie Lambert, Benoit

Pasquier, Véronique Mateo, Pauline Soulas, Lionel Galicier, et al. 2006. “XIAP Deficiency in Humans Causes an X-Linked Lymphoproliferative Syndrome.” *Nature* 444 (7115): 110–14. doi:10.1038/nature05257.

Roshan J Thapaa, Shoko Nogusaa, Peirong Chena, Jenny L Makib, Anthony Lerroa, Mark Andrakea, Glenn F Ralla, Alexei Degterevb, and Siddharth Balachandran. 2013. “Interferon-Induced RIP1/RIP3-Mediated Necrosis Requires PKR and Is Licensed by FADD and Caspases.” *Proceedings of the National Academy of Sciences of the United States of America*, July, 1–10. doi:10.1073/pnas.1301218110/-/DCSupplemental/pnas.201301218SI.pdf.

Salvesen, Guy S, and Colin S Duckett. 2002. “Apoptosis: IAP Proteins: Blocking the Road to Death's Door.” *Nature Reviews Molecular Cell Biology* 3 (6): 401–10. doi:10.1016/S0092-8674(00)00076-3.

Savage, Adam K, Michael G Constantinides, Jin Han, Damien Picard, Emmanuel Martin, Bofeng Li, Olivier Lantz, and Albert Bendelac. 2008. “The Transcription Factor PLZF (*Zbtb16*) Directs the Effector Program of the NKT Cell Lineage.” *Immunity* 29 (3): 391–403.

Schmid, J P, D Canioni, D Moshous, F Touzot, N Mahlaoui, F Hauck, H Kanegane, et al. 2011. “Clinical Similarities and Differences of Patients with X-Linked Lymphoproliferative Syndrome Type 1 (XLP-1/SAP Deficiency) Versus Type 2 (XLP-2/XIAP Deficiency).” *Blood* 117 (5): 1522–29. doi:10.1182/blood-2010-07-298372.

Schroeder, M A, and J F DiPersio. 2011. “Mouse Models of Graft-Versus-Host Disease: Advances and Limitations.” *Disease Models & Mechanisms* 4 (3): 318–33. doi:10.1242/dmm.006668.

Schweichel, J U, and H J Merker. 1973. “The Morphology of Various Types of Cell Death in Prenatal Tissues.” *Teratology* 7 (June): 253–66.

Scoltock, Alyson B, and John A Cidlowski. 2004. “Activation of Intrinsic and Extrinsic Pathways in Apoptotic Signaling During UV-C-Induced Death of Jurkat Cells: the Role of Caspase Inhibition.” *Experimental Cell Research* 297 (June): 212–23. doi:10.1016/j.yexer.2004.03.025.

Scott, Fiona L, Jean-Bernard Denault, Stefan J Riedl, Hwain Shin, Martin Renatus, and Guy S Salvesen. 2005. “XIAP Inhibits Caspase-3 and -7 Using Two Binding Sites: Evolutionarily Conserved Mechanism of IAPs .” *EMBO Journal* 24 (3): 645–55.

Segura, Elodie, Maxime Touzot, Armelle Bohineust, Antonio Cappuccio, Gilles Chiocchia, Anne Hosmalin, Marc Dalod, Vassili Soumelis, and Sebastian Amigorena. 2013. “Human Inflammatory Dendritic Cells Induce Th17 Cell Differentiation.” *Immunity* 38 (2). Elsevier Inc.: 336–48. doi:10.1016/j.immuni.2012.10.018.

Sherlock, Jonathan P, Barbara Joyce-Shaikh, Scott P Turner, Cheng-Chi Chao, Manjiri Sathe, Jeff Grein, Daniel M Gorman, et al. 2012. “IL-23 Induces Spondyloarthropathy by Acting on ROR-.” *Nature Medicine* 18 (7). Nature Publishing Group: 1069–76. doi:10.1038/nm.2817.

Shiozaki, Eric N, Jijie Chai, Daniel J Rigotti, Stefan J Riedl, Pingwei Li, Srinivasa M Srinivasula, Emad S Alnemri, Robert Fairman, and Yigong Shi. 2003. “Mechanism of XIAP-Mediated Inhibition of Caspase-9.” *Molecular Cell*, February, 1–9.

Shlomchik, Warren D. 2007. “Graft-Versus-Host Disease.” *Nature Reviews Immunology* 7 (5): 340–52. doi:10.4049/jimmunol.171.3.1272.

Silke, John, and Domagoj Vucic. 2014. *IAP Family of Cell Death and*

Signaling Regulators. *Regulated Cell Death Part B: Necroptotic, Autophagic and Other Non-Apoptotic Mechanisms*. 1st ed. Vol. 545. Methods in Enzymology. doi:10.1016/B978-0-12-801430-1.00002-0.

Silva-Santos, Bruno, Karine Serre, and Håkan Norell. 2015. “T Cells in Cancer.” *Nature Reviews Immunology* 15 (11). Nature Publishing Group: 683–91. doi:10.1038/nri3904.

Sneller, Michael C, Stephen E Straus, Elaine S Jaffe, Jonathan S Jaffe, Thomas A Fleischer, Maryalice Stetler-Stevenson, and Warren Strober. 1992. “A Novel Lymphoproliferative/Autoimmune Syndrome Resembling Murine Ipr/Gid Disease
” 90 (August): 1–8.

Speckmann, C, K Lehmberg, Albert Bendelac, R B Damgaard, M Fritsch, M Gyrd-Hansen, A Rensing-Ehl, et al. 2013. “X-Linked Inhibitor of Apoptosis (XIAP) Deficiency: the Spectrum of Presenting Manifestations Beyond Hemophagocytic Lymphohistiocytosis..” *Clinical Immunology (Orlando, Fla.)* 149 (1): 133–41. doi:10.1016/j.clim.2013.07.004.

Srenathan, Ushani, Kathryn Steel, and Leonie S Taams. 2016. “Immunology Letters.” *Immunology Letters* 178 (October). Elsevier B.V.: 20–26. doi:10.1016/j.imlet.2016.05.001.

Stephane Gerart, Sophie Sibe ril, Emmanuel Martin, Christelle Lenoir, Claire Aguilar, Olivier Lantz Capucine Picard, Alain Fischer, and Sylvain Latour. 2013. “Human iNKT and MAIT Cells Exhibit a PLZF-Dependent Proapoptotic Propensity That Is Counterbalanced by XIAP .” *Blood* 121 (4): 1–11. doi:10.1182/blood-2012-09.

Sumaria, Nital, Ben Roediger, Lai Guan Ng, Jim Qin, Rachel Pinto, Lois L Cavanagh, Elena Shklovskaya, Barbara Fazekas de St Groth, James A Triccas, and Wolfgang Weninger. 2011. “Cutaneous Immunosurveillance by Self-Renewing Dermal Γδ T Cells.” *Journal of Experimental Medicine* 208 (3): 505–18. doi:10.1074/jbc.273.12.7118.

Sun, Liming, Huayi Wang, Zhigao Wang, Sudan He, She Chen, Daohong Liao, Lai Wang, et al. 2012. “Mixed Lineage Kinase Domain-Like Protein Mediates Necrosis Signaling Downstream of RIP3 Kinase.” *Cell* 148 (1-2). Elsevier Inc.: 213–27. doi:10.1016/j.cell.2011.11.031.

Sutton, Caroline E, Stephen J Lalor, Cheryl M Sweeney, Corinna F Brereton, Ed C Lavelle, and Kingston H G Mills. 2009. “Interleukin-1 and IL-23 InduceInnate IL-17 Production From Gd T Cells, Amplifying Th17 Responses and Autoimmunity.” *Immunity* 31 (2). Elsevier Ltd: 331–41. doi:10.1016/j.immuni.2009.08.001.

Swain, Susan L. 1995. “Who Does the Polarizing?.” *Current Biology* 5 (8): 849–51.

Tait, Stephen W G, Gabriel Ichim, and Douglas R Green. 2014. “Die Another Way – Non-Apoptotic Mechanisms of Cell Death.” *Journal of Cell Science* 127 (10): 2135–44. doi:10.1242/jcs.093575.

Takahashi, Nozomi, Ineke Vanlaere, Riet de Rycke, Anje Cauwels, Leo A B Joosten, Erik Lubberts, Wim B van den Berg, and Claude Libert. 2008. “IL-17 Produced by Paneth Cells Drives TNF-Induced Shock.” *Journal of Experimental Medicine* 205 (8): 1755–61. doi:10.1084/jem.20061308.

Takeshi Kawabe, Dragana Jankovic, Shuko Kawabe, Yuefeng Huang, Ping-Hsien Lee, Hidehiro Yamane, Jinfang Zhu, Alan Sher, Ronald N Germain, and William E Paul. 2017. “Memory-Phenotype CD4+ T Cells Spontaneously Generated Under Steady-State Conditions Exert Innate

T_H1-Like Effector Function." *Science Immunology* 2 (12): 1–16.
Takeuchi, Osamu, and Shizuo Akira. 2010. "Pattern Recognition Receptors and Inflammation." *Cell* 140 (6): 805–20. doi:10.1016/j.cell.2010.01.022.
Tamm, Ingo, Stephan Richter, Doreen Oltersdorf, Ursula Creutzig, Jochen Harbott, Frank Scholz, Leonid Karawajew, Wolf-Dieter Ludwig, and Christian Wuchter. 2004. "High Expression Levels of X-Linked Inhibitor of Apoptosis Protein and Survivin Correlate with Poor Overall Survival in Childhood *De Novo* Acute Myeloid Leukemia ." *Clinical Cancer Research* 10 (June): 3737–44.
Taylor, Rebecca C, Sean P Cullen, and Seamus J Martin. 2008. "Apoptosis: Controlled Demolition at the Cellular Level." *Nature Reviews Molecular Cell Biology* 9 (3): 231–41. doi:10.1016/S0167-4838(98)00133-2.
Thornberry, N A, H G Bull, J R Calaycay, K T Chapman, A D Howard, M J Kostura, D K Miller, S M Molineaux, J R Weidner, and J Aunins. 1992. "A Novel Heterodimeric Cysteine Protease Is Required for Interleukin-1 Beta Processing in Monocytes.." *Nature* 356 (6372): 768–74. doi:10.1038/356768a0.
Thornberry, Nancy A, and Yuri Lazebnik. 1998. "Caspases: Enemies Within." *Science* 281 (August): 1312–16.
Trichonas, George, Yusuke Murakamia, Aristomenis Thanosa, Yuki Morizanea, Maki Kayamaa, Christine Debouckc, Toshio Hisatomia, Joan W Millera, and Demetrios Vavvas. 2010. "Receptor Interacting Protein Kinases Mediate Retinal Detachment-Induced Photoreceptor Necrosis and Compensate for Inhibition of Apoptosis ." *Proceedings of the National Academy of Sciences of the United States of America* 107 (50): 21695–700. doi:10.1073/pnas.1009179107/-/DCSupplemental.
Ueyama, Azumi, Chihiro Imura, Yasuyuki Fusamae, Kenichiro Tsujii, Yoko Furue, Miwa Aoki, Minoru Suzuki, et al. 2017. "Potential Role of IL-17-Producing CD4/CD8 Double Negative Ab T Cells in Psoriatic Skin Inflammation in a TPA-Induced STAT3C Transgenic Mouse Model ." *Journal of Dermatological Science* 85 (1). Japanese Society for Investigative Dermatology: 27–35. doi:10.1016/j.jdermsci.2016.10.007.
Upton, Jason W, William J Kaiser, and Edward S Mocarski. 2012. "DAI/ZBP1/DLM-1 Complexes with RIP3 to Mediate Virus-Induced Programmed Necrosis That Is Targeted by Murine Cytomegalovirus vIRA." *Cell Host & Microbe* 11 (3): 290–97. doi:10.1016/j.chom.2012.01.016.
Vandenabeele, Peter, Lorenzo Galluzzi, Tom Vanden Berghe, and Guido Kroemer. 2010. "Molecular Mechanisms of Necroptosis: an Ordered Cellular Explosion." *Nature Publishing Group* 11 (10). Nature Publishing Group: 700–715. doi:10.1038/nrm2970.
Varfolomeev, Eugene, Tatiana Goncharov, Anna V Fedorova, Jasmin N Dynek, Kerry Zobel, Kurt Deshayes, Wayne J Fairbrother, and Domagoj Vucic. 2008. "C-IAP1 and C-IAP2 Are Critical Mediators of Tumor Necrosis Factor A (TNFα)-Induced NF-κB Activation." *Journal of Biological Chemistry* 283 (36): 24295–99. doi:10.1016/S0092-8674(00)81064-8.
Vasconcelos, Nathalia M, Nina Opdenbosch, and Mohamed Lamkanfi. 2016. "Inflammasomes as Polyvalent Cell Death Platforms." *Cellular and Molecular Life Sciences* 73 (11). Springer International Publishing: 2335–47. doi:10.1007/s00018-016-2204-3.

Veillette, André, Luis-Alberto Pérez-Quintero, and Sylvain Latour. 2013. “X-Linked Lymphoproliferative Syndromes and Related Autosomal Recessive Disorders.” *Current Opinion in Allergy and Clinical Immunology* 13 (6): 614–22. doi:10.1097/ACI.0000000000000008.

Veldhoen, Marc, Richard J Hocking, Christopher J Atkins, Richard M Locksley, and Brigitta Stockinger. 2006. “TGFbeta in the Context of an Inflammatory Cytokine Milieu Supports De Novo Differentiation of IL-17-Producing T Cells..” *Immunity* 24 (2): 179–89. doi:10.1016/j.immuni.2006.01.001.

Villani, F N A, M O da Costa Rocha, M d C P Nunes, L R d V Antonelli, L M D Magalhaes, J S C dos Santos, K J Gollob, and W O Dutra. 2010. “Trypanosoma Cruzi-Induced Activation of Functionally Distinct ?? and ?? CD4- CD8- T Cells in Individuals with Polar Forms of Chagas' Disease.” *Infection and Immunity* 78 (10): 4421–30. doi:10.1128/IAI.00179-10.

Vince, James E, and John Silke. 2016. “The Intersection of Cell Death and Inflammasome Activation.” *Cellular and Molecular Life Sciences* 73 (11). Springer International Publishing: 2349–67. doi:10.1007/s00018-016-2205-2.

Vince, James E, Delara Pantaki, Rebecca Feltham, Peter D Mace, Stephanie M Cordier, Anna C Schmukle, Angelina J Davidson, et al. 2009. “TRAF2 Must Bind to Cellular Inhibitors of Apoptosis for Tumor Necrosis Factor (TNF) to Efficiently Activate NF- Band to Prevent TNF-Induced Apoptosis.” *The Journal of Biological Chemistry* 284 (51): 35906–15.

Wada, Taizo, Hirokazu Kanegane, Kazuhide Ohta, Fumiyo Katoh, Toshihiko Imamura, Yozo Nakazawa, Ritsuko Miyashita, et al. 2014. “Sustained Elevation of Serum Interleukin-18 and Its Association with Hemophagocytic Lymphohistiocytosis in XIAP Deficiency.” *Cytokine* 65 (1). Elsevier Ltd: 74–78. doi:10.1016/j.cyto.2013.09.007.

Walsh, Kevin P, and Kingston H G Mills. 2013. “Dendritic Cells and Other Innate Determinants of T Helper Cell Polarisation.” *Trends in Immunology* 34 (11). Elsevier Ltd: 521–30. doi:10.1016/j.it.2013.07.006.

Wan-Chen Hsieh, Ya-Ting Chuang, I-Hsuan Chiang, Shu-Ching Hsu, Shi-Chuen Miaw, and Ming-Zong Lai. 2014. “Inability to Resolve Specific Infection Generates Innate Immunodeficiency Syndrome in Xiap-/- Mice.” *Blood* 124 (18): 2847–57. doi:10.1182/blood-2014-03.

Wang, Huayi, Liming Sun, Lijing Su, Josep Rizo, Lei Liu, Li-Feng Wang, Fu-Sheng Wang, and Xiaodong Wang. 2014. “Mixed Lineage Kinase Domain-Like Protein MLKL Causes Necrotic Membrane Disruption Upon Phosphorylation by RIP3.” *Molecular Cell* 54 (1). Elsevier Inc.: 133–46. doi:10.1016/j.molcel.2014.03.003.

Wang, Lai, Fenghe Du, and Xiaodong Wang. 2008. “TNF-A Induces Two Distinct Caspase-8 Activation Pathways.” *Cell* 133 (4): 693–703. doi:10.1016/j.cell.2008.03.036.

Watarai, Hiroshi, Etsuko Sekine-Kondo, Tomokuni Shigeura, Yasutaka Motomura, Takuwa Yasuda, Rumi Satoh, Hisahiro Yoshida, et al. 2012. “Development and Function of Invariant Natural Killer T Cells Producing TH2- and TH17-Cytokines.” Edited by Philippa Marrack. *PLoS Biology* 10 (2): e1001255. doi:10.1371/journal.pbio.1001255.s013.

Wencker, Melanie, Gleb Turchinovich, Rafael Di Marco Barros, Livija Deban, Anett Jandke, Andrew Cope, and Adrian C Hayday. 2013. “Innate-Like T

Cells Straddle Innate and Adaptive Immunity by Altering Antigen-Receptor Responsiveness." *Nature Immunology* 15 (1): 80–87. doi:10.1002/cyto.a.10072.

Wéra, Odile, Patrizio Lancellotti, and Cécile Oury. 2016. "The Dual Role of Neutrophils in Inflammatory Bowel Diseases." *Journal of Clinical Medicine* 5 (12): 118. doi:10.1172/JCI57990.

Wilson, Michael T, Cecilia Johansson, Olivares-Villagomez Danyvid, Avneesh K Singh, Aleksandar K Stanic, Chyung-Ru Wang, Sebastian Joyce, Mary Jo Wick, and Luc Van Kaer. 2003. "The Response of Natural Killer T Cells to Glycolipidantigens Is Characterized by Surface Receptordown-Modulation and Expansion." *Proceedings of the National Academy of Sciences of the United States of America* 100 (19): 1–6.

Witowski, J, K Pawlaczyk, A Breborowicz, A Scheuren, M Kuzlan-Pawlaczyk, J Wisniewska, A Polubinska, et al. 2000. "IL-17 Stimulates Intraperitoneal Neutrophil Infiltration Through the Release of GRO Alpha Chemokine From Mesothelial Cells.." *Journal of Immunology (Baltimore, Md. : 1950)* 165 (10): 5814–21.

Wong, W W L, J E Vince, N Lalaoui, K E Lawlor, D Chau, A Bankovacki, H Anderton, et al. 2014. "cIAPs and XIAP Regulate Myelopoiesis Through Cytokine Production in an RIPK1- and RIPK3-Dependent Manner." *Blood* 123 (16): 2562–72. doi:10.1182/blood-2013-06-510743.

Yabal, Monica, Nicole Müller, Heiko Adler, Nathalie Knies, Christina J Groß, Rune Busk Damgaard, Hirokazu Kanegane, et al. 2014. "XIAP Restricts TNF- and RIP3-Dependent Cell Death and Inflammasome Activation.." *Cell Reports* 7 (6): 1796–1808. doi:10.1016/j.celrep.2014.05.008.

Yang, Xi, Toshio Miyawaki, and Hirokazu Kanegane. 2012. "SAP and XIAP Deficiency in Hemophagocytic Lymphohistiocytosis." *Pediatrics International* 54 (4): 447–54. doi:10.1111/j.1442-200X.2012.03683.x.

Yang, Yili, Shengyun Fang, Jane P Jensen, Allan M Weissman, and Jonathan D Ashwell. 2000. "Ubiquitin Protein Ligase Activity of IAPs and Their Degradation in Proteasomes in Response to Apoptotic Stimuli." *Science* 288 (May): 874–77.

Yoneyama, Mitsutoshi, and Takashi Fujita. 2010. "Recognition of Viral Nucleic Acids in Innate Immunity." *Reviews in Medical Virology* 20 (1): 4–22. doi:10.4049/jimmunol.181.9.6427.

Young, K J. 2002. "Donor-Lymphocyte Infusion Induces Transplantation Tolerance by Activating Systemic and Graft-Infiltrating Double-Negative Regulatory T Cells." *Blood* 100 (9): 3408–14. doi:10.1182/blood-2002-01-0235.

Young, K J, B DuTemple, M J Phillips, and L Zhang. 2003. "Inhibition of Graft-Versus-Host Disease by Double-Negative Regulatory T Cells." *The Journal of Immunology* 171 (1): 134–41. doi:10.4049/jimmunol.171.1.134.

Young, Kevin J, and Li Zhang. 2002. "The Nature and Mechanisms of DN Regulatory T-Cell Mediated Suppression." *Human Immunology* 63 (September): 926–34.

Yuan, Junying, Ayaz Najafov, and Bénédicte F Py. 2016. "Roles of Caspases in Necrotic Cell Death." *Cell* 167 (7). Elsevier Inc.: 1693–1704. doi:10.1016/j.cell.2016.11.047.

Yvonne Zeissig, Britt-Sabina Petersen, Snezana Milutinovic, Esther Bosse, Gabriele Mayr, Kenneth Peuker, Jelka Hartwig, et al. 2014. "XIAP Variants in Male Crohn's Disease." *Gut* 64 (December): 66–76.

doi:10.1136/gutjnl-2013-306520.
Zhang, Ting, Yan Zhang, Mingyao Cui, Li Jin, Yimei Wang, Fengxiang Lv, Yuli Liu, et al. 2016. “CaMKII Is a RIP3 Substrate Mediating Ischemia- and Oxidative Stress-Induced Myocardial Necroptosis.” *Nature Medicine* 22 (2). Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.: 175–82. doi:10.1038/nm.4017.
Zhang, Yutao, Jianhua Zhu, Yun Tang, Feng Li, Hongyuan Zhou, Bofang Peng, Chifeng Zhou, and Rong Fu. 2011. “X-Linked Inhibitor of Apoptosis Positive Nuclearlabeling: a New Independent Prognosticbiomarker of Breast Invasive Ductal Carcinoma.” *Diagnostic Pathology* 6 (1). BioMed Central Ltd: 49. doi:10.1186/1746-1596-6-49.
Zhang, Zhu, Kevin Young, and Li Zhang. 2001. “$Cd3^{+}Cd4^{-}Cd8^{-}$Aβ-TCR^{+} T Cell as Immune Regulatory Cell.” *Journal of Molecular Medicine* 79 (8): 419–27. doi:10.1007/s001090100238.
Zheng, L, N Bidere, D Staudt, A Cubre, J Orenstein, F K Chan, and M Lenardo. 2006. “Competitive Control of Independent Programs of Tumor Necrosis Factor Receptor-Induced Cell Death by TRADD and RIP1.” *Molecular and Cellular Biology* 26 (9): 3505–13. doi:10.1128/MCB.26.9.3505-3513.2006.
Zhu, Jinfang, Hidehiro Yamane, and William E Paul. 2010. “Differentiation of Effector CD4 T Cell Populations.” *Annual Review of Immunology* 28 (1): 445–89. doi:10.1146/annurev-immunol-030409-101212.

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BERUFLICHER WERDEGANG:

04/2018 - heute — FACHSEMINAR Life Science Management bei der ATV GmbH, Inhalte u.a.:

- Qualitätsmanagement (GxP)
- Projektmanagement
- Klinische Studien
- Zulassung und Zusatznutzen

11/2011 – 10/2017 — WISSENSCHAFTLICHER MITARBEITER am Klinikum rechts der Isar, Hämato/Onko

- Projektplanung und wissenschaftliche Recherche, Präsentation
- experimentelle Mausmodelle inkl. Analysen
- multi-color Durchflusszytometrie
- Primäre Zellkultur, Zelldifferenzierung (APCs und T Zellen)
- Zytokinbestimmung mit CBA und ELISA
- Protein- und RNA-Nachweis (Western Blot, Histologie, qPCR)
- Viability- und Proliferationsassays

12/2010 – 07/2011 — WISSENSCHAFTLICHE HILFSKRAFT am Universitätsklinikum Erlangen, Abteilung für experimentelle Tumorpathologie

- quantitative real-time PCR
- Western Blotting (inkl. cytoplas. und nukleären Extraktionen)
- ELISA
- ChIP
- Zellkultur von Zelllinien
- Proliferationsassays
- Bisulfitkonversion, methylierungsspezifische PCR, Pyrosequenzierung

08/2010 – 10/2010 — STIPENDIUM FÜR EIN PRAKTIKUM am MD Anderson Cancer Center der University of Texas/Houston, Department Molecular Oncology bei Prof. Dr. Elsa Flores

- Western Blotting
- quantitative real-time PCR und Primeretablierung
- Gewinnung und Kultivierung embryonaler Primärzellen
- Southern Blotting

05/2010 – 07/2010 — WISSENSCHAFTLICHE HILFSKRAFT an der Universität Erlangen, Abteilung für molekulare Pflanzenphysiologie

- Klonierung
- Konfokalmikroskopie

08/2009 – 09/2009 PRAKTIKUM am Helmholtz Institut München, Abteilung für molekulare Genetik bei Prof. Dr. Ursula Zimber-Strobl
- Transfektion
- Immunhistologische Probenvorbereitung und Färbung
- Mausgenotypisierung und Organpräparation
- Immunoblotting
- B-cell Isolation

08/2008 – 09/2008: PRAKTIKUM im Kreiskrankenhauslabor Vilsbiburg
- rountinemäßige Untersuchungen von Blut und Urinproben
- Blutgruppenbestimmung und Kreuzprobe

07/2007 – 08/2007 PRAKTIKUM bei Ovofit, Neumarkt-St.Veit
- Qualitätssicherung und Qualitätsmanagement
- Produktentwicklung

08/2006, 2 Wochen PRAKTIKUM bei U3 Pharma, Martinsried

AUSBILDUNG:

11/2011 – 10/2017 PROMOTION in Immunbiologie, Klinikum rechts der Isar, III. Medizinische Klinik, Arbeitsgruppe PD Dr. Philipp Jost
- „Characterization of the role of XIAP in modulating inflammatory death and its consequences"

12/2010 – 07/2011 MASTERARBEIT in der AG Experimentelle Tumorpathologie am Universitätsklinikum Erlangen
- „Epigenetische Veränderungen nach TNF-Behandlung von normalen humanen intestinalen Epithelzellen als Modellsystem für colitis ulcerosa"
- Abschlussnote: 1,3

10/2009 – 07/2011 MASTERSTUDIUM an der Friedrich-Alexander-Universität Nürnberg/Erlangen
- „Molekular- und Zellbiologie"
- „Master of Science", Abschlussnote 1,2

07/2009 – 08/2009 BACHELORARBEIT an der Paris-Lodron-Universität Salzburg
- „Lipidstoffwechsel – Störungen des Lipoproteinmetabolismus und Atherosklerose als eine der Folgen"
- Abschlussnote: 1

08/2008 – 09/2008 BACHELORARBEIT an der Paris-Lodron-Universität Salzburg
- „Structure-function relationships of stromal interaction molecule 1 (STIM1), as well as STIM1 – ORAI interactions evaluated by FRET microscopy"
- Abschlussnote: 1

10/2006 – 09/2009 BACHELORSTUDIUM an der Paris-Lodron-Universität Salzburg
- „Molekulare Biowissenschaften"
- „Bachelor of Science" mit Auszeichnung bestanden

01/2006 – 04/2006 SPRACHKURS an der University of Cork "English as a foreign language"

09/1996 – 07/2005 GYMNASIUM in Mühldorf am Inn

FREMDSPRACHEN UND IT SKILLS:

Englisch	fließend, Cambridge Advanced Certificate: Grade A
Französisch	Grundkenntnisse
Spanisch	Grundkenntnisse
Polnisch	Hörverständnis
IT-Kenntnisse:	Adobe Illustrator/Photoshop/Lightroom, GraphPad Prism, FlowJo, Microsoft Word/Exel/ Powerpoint

PUBLIKATIONEN UND AUSZEICHNUNGEN:

Blood Advances (submitted): "XIAP deficiency in hematopoietic recipient cells drives donor T cell activation and GvHD in mice", Nicole Müller, Julius C. Fischer, Monica Yabal, Tobias Haas, Hendrik Poeck and Philipp J. Jost

01/2017: Posterpreis "RIPK3 dependent cell death initiates IL-1 driven IL-17 induction in Th17 cells and innate T cell subsets"; EMBO conference " Cell death, inflammation and cancer" in Obergurgl, AT

06/2016: Leukemia Re-activation of mitochondrial apoptosis inhibits T-cell lymphoma survival and treatment resistance, Spinner S, Crispatzu G, Yi JH, Munkhbaatar E, Mayer P, Höckendorf U, Müller N, Li Z, Schader T, Bendz H, Hartmann S, Yabal M, Pechloff K, Heikenwalder M, Kelly GL, Strasser A, Peschel C, Hansmann ML, Ruland J, Keller U, Newrzela S, Herling M, Jost PJ

06/2014: Cell Reports XIAP Restricts TNF- and RIP3-Dependent Cell Death and Inflammasome Activation**,** Monica Yabal, Nicole Müller, Heiko Adler, Nathalie Knies, Christina J. Groß, Rune Busk Damgaard, Hirokazu Kanegane, Marc Ringelhan, Thomas Kaufmann, Mathias Heikenwälder, Andreas Strasser, Olaf Groß, Jürgen Ruland, Christian Peschel, Mads Gyrd-Hansen, and Philipp J. Jost

02/2014: PNAS ΔNp63 induces terminal differentiation through transcriptional regulation of DGCR8 and suppression of pluripotency factors, Deepavali Chakravart, Xiaohua Su, Min Soon Cho, Ngoc Hoang Bao Bui, Cristian Coarfac, Avinashnarayan Venkatanarayana, Ashley L. Benham, Ramón E. Flores González, Jennifer Alana, Weimin Xiao, Marco L. Leung, Harina Vin, Io Long Chan, Arianexys Aquino, Nicole Müller, Hongran Wang, Austin J. Cooney, Jan Parker-Thornburg, Kenneth Y. Tsai, Preethi H. Gunaratne, and Elsa R. Flores