Apoptotic and inflammatory signalling pathways in dendritic cells

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Summary

The immune system recognizes and eliminates infiltrating microorganisms and other pathogens like viruses and parasites. Beside monocytes, macrophages and B-lymphocytes, dendritic cells (DC) belong to the antigen-presenting cells (APC) and serve as point of intersection between the innate and adaptive immune system. DC recognition of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) by specific PAMP recognition receptors (PRRs) leads to their maturation and an upregulation of different molecules, e.g. the inflammatory cytokine IL-1β and the anti-apoptotic molecule cFLIP (cellular-FLICE inhibitory protein).

The aim of this thesis was the detailed characterisation of the respective inflammatory and apoptotic signalling pathways in murine DCs also by interference via either dominant-negative protein expression or siRNA-mediated knockdown and the analysis of their impact on T cell priming.

Beside DC-maturation, lipopolysaccharide, lipoteichonic acid, hyaluronic acid or a cocktail containing TNF-α, IL-4, CD40L and PGE2 together led to an activation of the inflammasome. The mRNA expression of IL-18, IL-33 and IL-1β and the protein expression of the inflammatory caspase-11 increased and IL-18 and IL-1β were secreted. The IL-1β secretion could be further increased by supplemental stimulation with the DAMP ATP, was dependent on caspase-activation as well as potassium gradient and regulated via the $P2X_7$ receptor. DC inflammasome activation did not influence the CD4+ TC proliferation rate, but showed a complex spectrum of induced cytokines, namely TNF-α, IL-2, IL-4, IL-10, GM-CSF, IL-6, IL-5, M-CSF and IL-17 arguing for a complex immune response dependent on the infection background. Expression of a dominant-negative variant of the adapter molecule ASC (dnASC) to block the inflammasome-mediated signalling cascade led to a massive DC death, and did not allow conclusions about the role of inflammatory caspases and their intracellular network in respect to the inflammatory cytokines.

DC maturation-induced upregulation of cFLIP inhibited CD95L-induced apoptosis by blocking caspase-8 at the death inducing signalling complex (DISC). A complete genetic knockout of cFLIP in DCs led to a severe spontaneous cell death rescued by expression of one cFLIP-allele. A knockdown of cFLIP also directly diminished DC lifespan, but had no specific influence on TC proliferation rate and DC survival during DC-TC interaction.

These findings provide new insights in the fundamental roles of ASC and cFLIP for the lifespan of DCs and may help to further develop the DC-based immunotherapy.

Zusammenfassung

Das Immunsystem erkennt und eliminiert eindringende Mikroorganismen und andere Pathogene wie Viren und Parasiten. Dendritische Zellen (DZ) zählen neben Monozyten, Makrophagen und B-Lymphozyten zu den professionellen Antigen-präsentierenden Zellen (APC) und fungieren als Schnittstelle zwischen dem angeborenen und dem erworbenen Immunsystem. Das Erkennen fremder molekularer Muster unterschiedlicher Pathogene (*pathogen-associated molecular patterns,* PAMPs) und eigener Gefahr-assoziierter Muster (*danger-associated molecular patterns*, DAMPs) über spezifische Rezeptoren (PAMP recognition receptors (PRRs)) führt bei DZ zur Reifung und einer Hochregulation verschiedener Moleküle, wie z.B. dem inflammatorischen Zytokin IL-1β und dem anti-apoptotischen cFLIP (cellular-FLICE inhibitory protein).

Das Ziel der Dissertation ist die detaillierte Charakterisierung der zugehörigen inflammatorischen und apototischen Signalwege in murinen DZ, des Weiteren auch durch Interferenz mittels dominant-negativer Proteinexpression bzw. siRNA-vermitteltem Knockdown und deren Auswirkungen auf das T-Zell-Priming.

Neben einer DZ-Reifung führen Lipopolysaccharid, Lipoteichonsäure, Hyaluronsäure oder ein Cocktail aus TNF-α, IL-4, CD40L und PGE2 zur Aktivierung des Inflammasoms. Dies führt zu einer gesteigerten Genexpression von IL-18, IL-33 und IL-1β und erhöhter Proteinexpression der inflammatorischen Caspase-11 und zur Freisetzung von IL-18 und IL-1β. Die IL-1β Freisetzung konnte über eine zusätzliche Stimulation mit dem DAMP ATP erhöht werden, war abhängig von der Caspase-Aktivität und einem Kaliumgradienten, und über den $P2X_7$ Rezeptor reguliert. Die Aktivierung des Inflammasoms in DZ hatte keinen Einfluss auf die CD4+ T-Zell-Proliferationsrate, zeigte aber ein komplexes Spektrum induzierter Zytokine, wie TNF-α, IL-2, IL-4, IL-10, GM-CSF, IL-6, IL-5, M-CSF und IL-17, welches für eine komplexe Immunantwort in Abhängigkeit vom Infektionshintergrund spricht. Die Expression einer dominant-negativen Form des Adaptermoleküls ASC (dnASC), welche den inflammatorischen Signalweg blockieren sollte, führte zu einem massiven DZ-Sterben und ließ keine Rückschlüsse über die Rolle der inflammatorischen Caspasen und ihr intrazelluläres Netzwerk im Hinblick auf die inflammatorischen Zytokine zu.

Die durch DZ-Reifung induzierte Hochregulation des Apoptose-Inhibitors cFLIP führt zu einer Resistenz gegenüber der CD95L-induzierten Apoptose durch die Inhibition der Caspase-8 im CD95 Rezeptorkomplex (DISC). Ein kompletter genetischer Verlust von cFLIP führte zu einem spontanen DZ-Zelltod, welcher bereits durch das Vorhandensein eines cFLIP-Allels verhindert werden konnte. Eine Verringerung der cFLIP Expression in DZ führte ebenso zu einer Verkürzung ihrer Lebensdauer, zeigte aber keinen spezifischen Einfluss auf die TZ-Proliferationsrate und das Überleben der DZ im Interaktionsmodell.

Diese Befunde erlauben einen tieferen Einblick in die fundamentalen Rollen von ASC und cFLIP in dendritischen Zellen und helfen möglicherweise bei der Weiterentwicklung der DZ-basierten Immuntherapie.

Content

1. Introduction

1.1. The immune system

1.1.1. Physiological function of the immune system

The immune system of an organism is a dynamic and complex network that recognizes and responds to tissue damage, pathogenic microbes, viruses and parasites after infection or injury. It is classified into innate and adaptive branches of the immune system, both containing cellular and humoral mechanisms and collaborating tightly.

The innate immune system with natural protection mechanisms is the phylogenetic older part of the immune system (Janeway, Jr. et al. 2012; Naito 1993; Ratcliffe et al. 1991; Takeda et al. 2003). The first line of host defence after the natural barrier of the skin is provided by the complement system and the unspecific cellular defence mediated by granulocytes, monocytes, macrophages, neutrophils, dendritic cells and natural killer cells.

The initiation of the adaptive immune response is the uptake and processing of foreign antigens by antigen presenting cells (APC) like dendritic cells. Fragments of the antigen in combination with host major histocompatibility complex (MHC) - molecules and costimulatory factors such as CD80 and CD86 are then presented by APCs to T cells (TC). This process leads to an activation of the antigen specific immune response followed by clonal proliferation of antigen specific lymphocytes and differentiation to effector cells like cytotoxic $CD8^+$ TCs or $CD4^+$ TCs. Cytokines, enzymes and acute-phase-proteins support the innate as well as the antigen-specific cellular defence (Janeway, Jr. et al. 2012).

1.1.2. Function of dendritic cells

Dendritic cells (DCs), the last discovered immune cell population and first described by Steinman and Cohn, are professional APCs and of significant relevance in activating the immune response (Steinman et al. 1975; Steinman and Cohn 1973; Steinman and Cohn 1974). Briefly, they gather antigens, present those on their surface and induce an immune response by activation of T-cell receptors and production of cytokines. Thus, DCs serve as a point of intersection between the innate and adaptive immune system by connecting phagocytosis and antigen presentation (Janeway, Jr. et al. 2012).

DCs are a heterogeneous cell population and can be divided into different highly specialized subpopulations for example myeloid/conventional- and plasmacytoid DCs (Collin and Bigley 2018; Janeway, Jr. et al. 2012). The various DC subtypes are derived from $CD34^+$ bone marrow precursor cells (Caux et al. 1997; Romani et al. 1994). In particular, myeloid precursor cells differentiate into interstitial DCs in organs and tissues whereas lymphoid precursor cells become plasmacytoid DCs localized in lymphatic tissues. A part of DCs circulate in blood and lymphatic vessels (Knight et al. 1982; Spry et al. 1980) and thus represent 1 % of the peripheral mononuclear cells. DCs remain in tissues and organs until finding and gathering an antigen by different mechanisms like macropinocytosis, receptor-mediated endocytosis and phagocytosis. DC antigen uptake by receptor-mediated endocytosis occurs by adsorption of antigens at receptors, e.g. a combined inclusion of antigens. DCs express several of those receptors for example the Fc-receptors (FcR) (Banchereau and Steinman 1998; Fanger et al. 1996), the macrophage antigen 1 (Mac-1) molecule also known as CD11b/CD18 complex or αMβ2 integrin (Anderson et al. 1986), C type lectin receptor type 1 DEC-205 (Jiang et al. 1995), the mannose receptor (Tan et al. 1997), CD14 (Rescigno et al. 1999) and the Toll-like receptors (TLR) (Medzhitov 2001; Poltorak et al. 1998a; Poltorak et al. 1998b; Yang et al. 1998).

Apoptotic and necrotic cell fragments, also viruses, bacteria and parasites are gathered by phagocytosis for example via CD36, αvβ3- or αvβ5-integrins. Pathogenic intracellular molecules for example DNA with non-methylated CpG-motives, which often appear in the genome of bacteria and viruses, are recognized by the

cytoplasmic nucleotide-binding and oligomerization domain proteins (NODs) (Philpott and Girardin 2004). Molecules and molecule compositions characteristic for a broad spectrum of microorganisms like lipopolysaccharide (LPS) or peptidoglycan (PGN) are called pathogen associated molecular patterns (PAMPs) and host molecules like ATP released upon injury or infection are called danger associated molecular patterns (DAMPs) (Janeway, Jr. et al. 2012). It is hypothesized that the organism distinguishes between dangerous and harmless antigens by using those receptors (Matzinger 1998).

Antigen uptake and processing, triggering of TLR by ligands, interaction with other molecules like CD40 and CD40L or the presence of cytokines induce a line of phenotypical, morphological and functional changes in immature DCs (Jonuleit et al. 1997; Reddy et al. 1997; Sallusto et al. 1995). Transition from an antigen incorporating cell to an antigen presenting cell is characterized by the loss of the ability to take up and process an antigen. Simultaneously, this process is accompanied by the increased expression of MHC class I and MHC class II molecules as well as different costimulatory and adhesion molecules, e.g. CD54, CD80, CD86 and CD40, which are essential for TC activation on the cell surface.

Upon antigen uptake, DCs migrate to lymph nodes, present the assimilated and processed antigen to naive TCs and initiate their differentiation to effector cells (Inaba et al. 1993). This process is influenced by signals transmitted by the DC, the concentration of the antigen as well as cytokines and other soluble factors of the environment. The cell-cell contact is mediated by interaction of adhesion molecules, thus generating the so-called immunological synapse. DC adhesion molecules are mainly CD11c, CD54 (ICAM-1), CD102 (ICAM-2), DC-SIGN and CD58 (LFA-3). TC adhesion molecules include CD18, CD50 (ICAM-3), CD11a (LFA-1) and CD2 (LFA-2) (Banchereau and Steinman 1998; Bell et al. 1998; Ganpule et al. 1997; Hauss et al. 1995; Inaba et al. 1989; Prickett et al. 1992; Xu et al. 1992) (Figure 1, adhesion). Specific binding of a TC receptor to an antigen/MHC-complex can augment the affinity of the adhesion molecules so that cells stay in contact for two to three hours (Catron et al. 2004; Dustin and Springer 1989).

Mature DCs can stimulate naive $CD4^+$ TCs (antigen presented by MHC-II) as well as naive CD 8^+ TCs (antigen on MHC-I). For a complete TC activation a further

costimulatory signal is needed like binding of DC molecules CD40, CD80 or CD86 to the appropriate TC molecules CD40L or CD28 (Figure 1, binding and costimulation) (Caux et al. 1994; Gonzalo et al. 2001; Inaba et al. 1994). TCs become anerg or go into apoptosis if a costimulatory signal is missing (Jenkins 1994; Schwartz 1990).

Upon DC-TC cell contact, Interleukin (IL-) 2 is produced and the IL-2 receptor is upregulated, leading to clonal expansion of TCs (Fraser et al. 1991; Jain et al. 1995). Naive $CD8^+$ TCs e.g., activated by an MHC-class I bound antigen, differentiate into cytotoxic TC (CTL). Those CTLs attack the recognized cell with perforin and granzymes, thereby causing apoptosis by perforating the membrane of the attacked cell, or activating the Fas-receptor (Andersen et al. 2006; O'Rourke and Mescher 1992; Smyth and Trapani 1995).

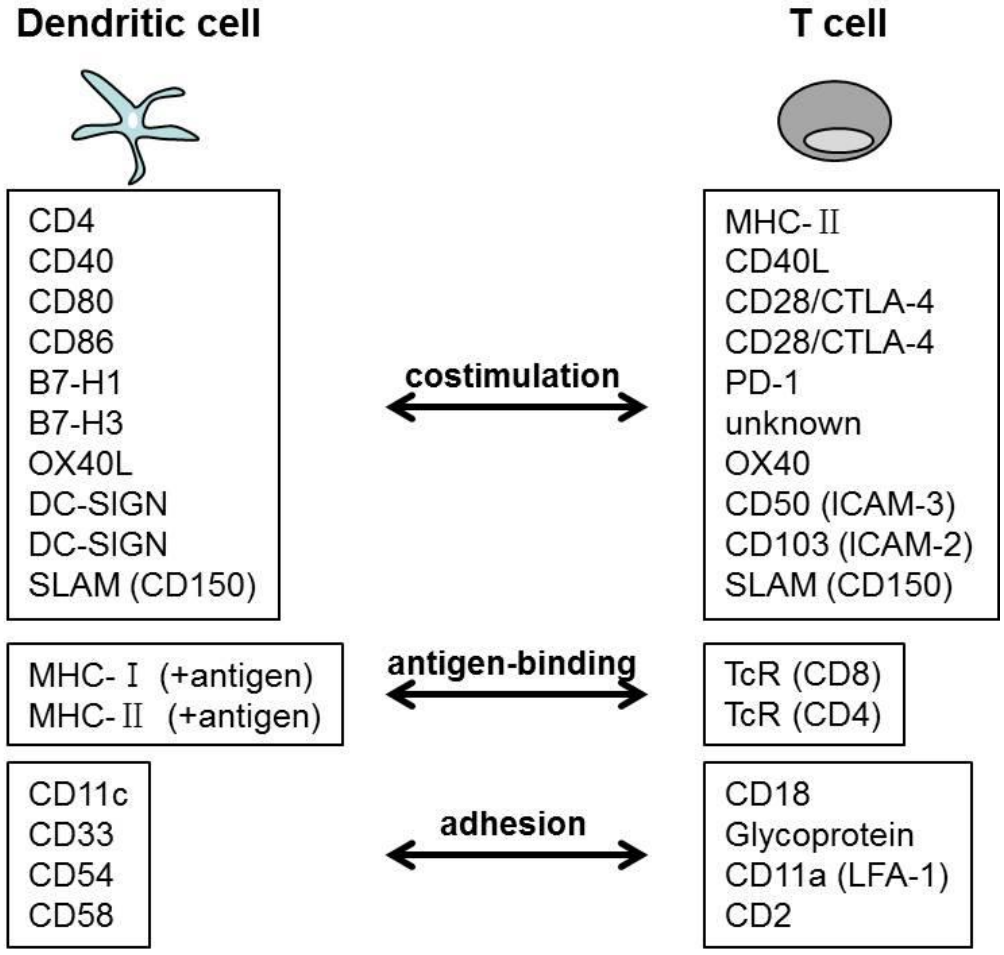


Figure 1: Molecules involved for activation of naïve TCs by DCs.
The required molecules for activation of naïve TCs by DCs are depicted. In detail, adhesion molecules are essential for maintenance of cell-cell contacts. The antigens are presented on MHC-molecules. TCs require a second signal essential for their activation by costimulatory molecules located only on APCs (Beck 2003).

The secretion of cytokines plays an important role during differentiation of antigen/MHC-II complex activated $CD4^+$ TC (Mosmann and Coffman 1989a; Mosmann and Coffman 1989b). In the presence of IL-12, the activated $CD4^+$ TC differentiates into a T-helper cell type 1 (Th1) secreting pro-inflammatory cytokines like granulocyte macrophage costimulatory factor (GM-CSF), interferon (IFN) -γ, IL-2 and tumor necrosis factor (TNF) -α and thus activating, among others, macrophages. In the presence of IL-4, IL-6 and IL-10, the $CD4^+$ TC differentiates into T-helper cell type 2 (Th2). These cells secrete prevalent anti-inflammatory and B-cell stimulatory cytokines like IL-2, IL-4, IL-5, IL-6, TGF-β and IL-10 thereby stimulating B-cell antibody production (Paul et al. 1993a; Paul et al. 1993b). Independent of the antigen, but dependent on cytokine environment the Th1- or/and Th2 response is initiated. If both are initialized Th1 response starts and upon some hours a change of cytokine production leads to the Th2 response (Langenkamp et al. 2000). IL-12 promotes Th1 polarization whereas IL-4 provokes a Th2 polarization (Seder and Paul 1994; Trinchieri and Scott 1995). Length and intensity of antigen stimulation (Constant et al. 1995; Iezzi et al. 1999), type of costimulatory molecules (Kuchroo et al. 1995) as well as the DC maturation status (Langenkamp et al. 2000) influence significantly the result of TC polarization. In Figure 2 the different ways of TC activation with the involved molecules are shown.

DCs are also able to induce regulatory TCs (Jonuleit et al. 2001; Yamazaki et al. 2003), which inhibit TC activation (Mason and Powrie 1998; Sakaguchi 2000; Shevach 2000). Furthermore, they can activate IL-17 producing $CD4^+$ TCs. It has been demonstrated that IL-1 (α and β) and IL-23 have a crucial role in the induction of IL-17-producing TCs (Zheng et al. 2007). A modulated cytokine secretion pattern (IL-1 and IL-23) influences memory TC response and mediates inflammatory pathology in a number of certain autoimmune diseases. Plasmacytoid DCs produce not only IL-12 but also a large amount of IFN-γ, which inhibits viral replication, enhances the MHC-class- I production and activates natural killer cells (NK-cells) (Paul et al. 1993b).

Upon antigen presentation DCs undergo apoptosis after approximately 72 hours of stimulation (Catron et al. 2004; Ingulli et al. 1997; Kamath et al. 2000). DC apoptosis regulates the balance between tolerance and immunity and is self-regulated by extrinsic and T-cell-mediated signals (Kushwah and Hu 2010; Oh and Shin 2015). If

apoptosis induction of DC in lymph nodes is prevented by inhibition of central proteases of the apoptotic signalling cascade, DCs accumulate in lymph nodes leading to autoimmunity. This emphasizes the significance of DCs apoptosis in completing immune responses (Bouillet and Strasser 2002; Chen et al. 2006c; Chen et al. 2007; Cohen and Eisenberg 1992).

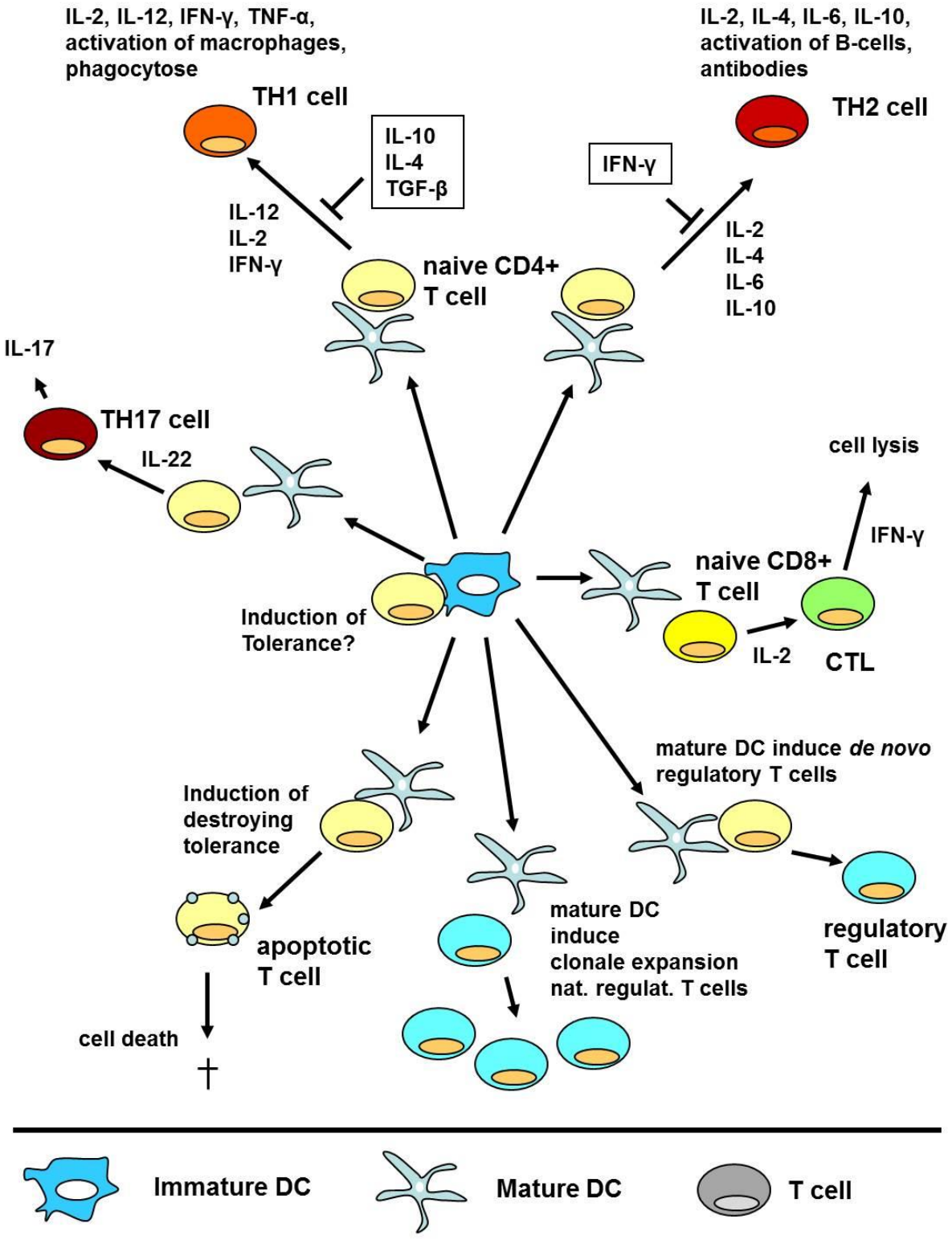

IL-2, IL-12, IFN-γ, TNF-α,
activation of macrophages,
phagocytose
TH1 cell
IL-10
IL-4
TGF-β
IL-12
IL-2
IFN-γ
IL-2, IL-4, IL-6, IL-10,
activation of B-cells,
antibodies
TH2 cell
IFN-γ
IL-2
IL-4
IL-6
IL-10
naive CD4+
T cell
IL-17
TH17 cell
IL-22
cell lysis
IFN-γ
naive CD8+
T cell
Induction of
Tolerance?
IL-2
CTL
mature DC induce *de novo*
regulatory T cells
Induction of
destroying
tolerance
apoptotic
T cell
cell death
mature DC
induce
clonale expansion
nat. regulat. T cells
regulatory
T cell
Immature DC
Mature DC
T cell

Figure 2

Figure 2: Role of DC and the cytokine spectrum for activation and regulation of immune cells.
In response to environmental signals immature DCs can mature to multiple types of 'effector' DCs that instruct distinct T-cell fates, including immunity, tolerance and immune deviation. Maturation signals can be of exogenous (for example microbial) or endogenous (for example cytokines, hormones and dying cells) origin. The quality of these signals largely determines the choice of effector DC. Some signals can promote the generation of tolerogenic DCs and some immature DCs have an intrinsic tolerogenic function.
Abbreviations: cytotoxic T lymphocyte (CTL); interferon-γ (IFN-γ); interleukin- (IL-); transforming growth factor β (TGF-β); T helper (TH); tumor necrosis factor α (TNF-α) (adapted from (Reis e Sousa 2006))

1.1.3. The inflammasome

Cytokines activate, trigger or repress signalling pathways during inflammatory processes and immune responses. The interleukin 1 family cytokines are expressed as preforms and the inflammasome is the molecular platform for processing and maturing these cytokines such as interleukin-1, -18, and -33 (IL-1, IL-18, IL-33) (Arend et al. 2008; Martinon et al. 2002). Cleaved cytokines along with other components of the inflammasome can be released by the cell (Ogura et al. 2006).

Inflammasomes contain one or two members of inflammatory caspases and an intracellular receptor of the nucleotide oligomerization domain (NOD) – like receptor family (NLR). Furthermore the adaptor proteins ASC and CARDINAL can be involved in molecular composition of this protein complex (Figure 3) (Harton et al. 2002). Homotypic interaction of certain domains of involved proteins followed by oligomerization of multiple molecule complexes via oligomerization domains results in a stereoscopic concentration of caspase- molecules and their proteolytic cleavage. Inflammatory protein complexes itself can differ in their composition due to the multitude of molecules of inflammasome components, the expression profile or the availability of PAMPs (Delbridge and O'Riordan 2007; Mariathasan et al. 2006). Binding of a specific ligand like muramyldipeptide (MDP) on the leucine rich repeat (LRR) domain of NACHT, LRR and PYD domains-containing protein 3 (NALP3) for example leads to activation of the NALP3 inflammasome (Martinon et al. 2004). Oligomerization of multiple complexes via the nucleotide-binding site (NBS) domain

activates pro-caspase-1 and catalyzes the proteolytic processing of the cytokine IL-1β (Figure 3).

The activated caspases cleave pro-peptides of the before mentioned cytokines in their active form. Human Caspase-1, -4, -5 and murine Caspase-1, -11 and -12 belong to these inflammatory caspases. The best-known caspase is caspase-1, which is important for the proteolytic maturation of the pro-inflammatory cytokine IL-1β (Cerretti et al. 1992; Thornberry et al. 1992). Inflammasomes counter viral replication and remove infected cells through an inflammatory cell death called pyroptosis (Cridland et al. 2012; Gram et al. 2012; Lamkanfi and Dixit 2014; Martinon et al. 2002). Dysregulation of the inflammasome for example by mutations in a gene can lead to hereditary autoimmune diseases like the Muckle-Wells-syndrome (MWS), the Familial Cold Autoinflammatory Syndrome and Neonatal Onset Multisystem Inflammatory Disease (NOMID) (Agostini et al. 2004; Hoffman et al. 2004; Neven et al. 2004).

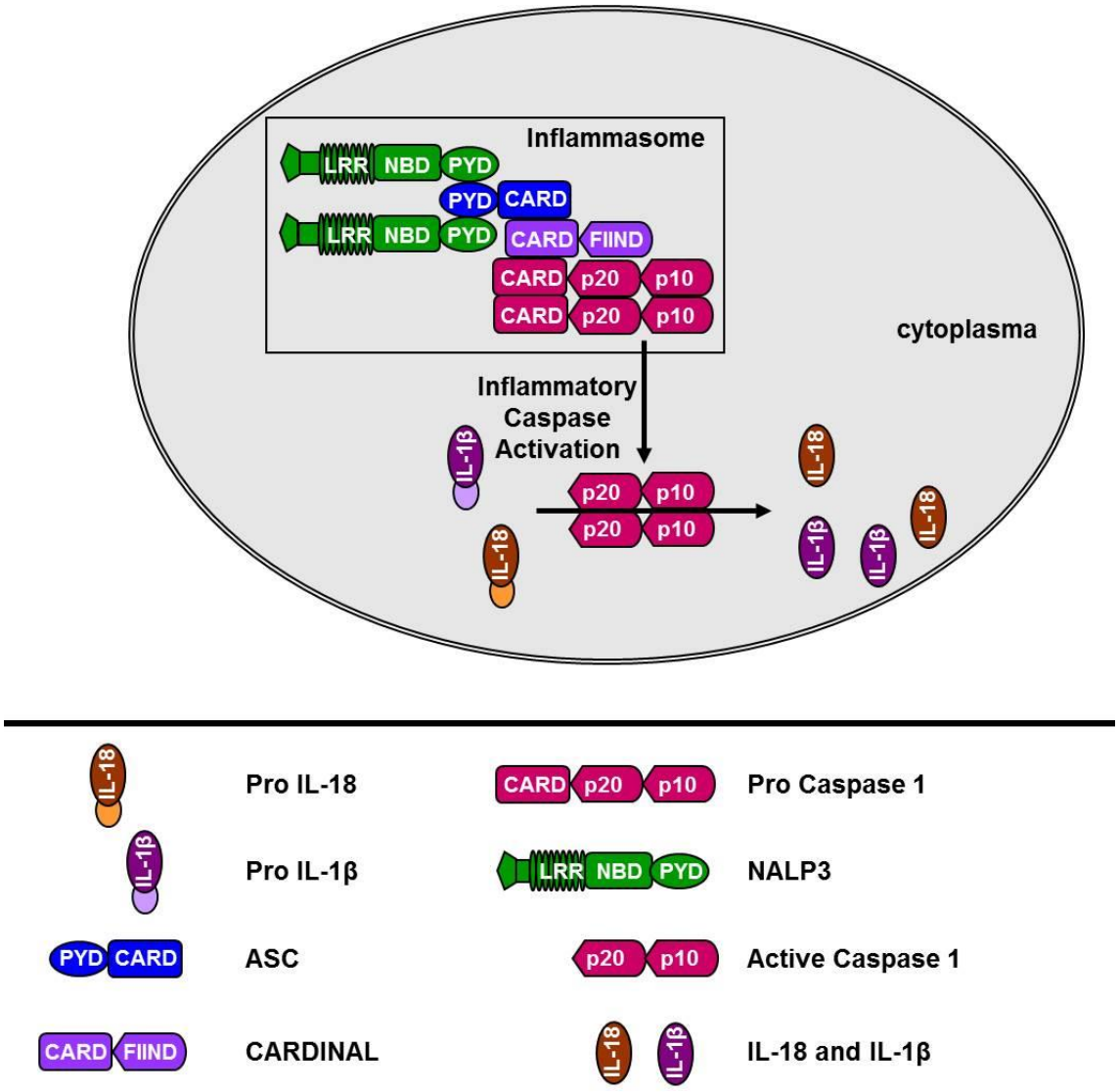


Figure 3

Figure 3: Scheme of the role of the Inflammasome for caspase-mediated cytokine processing (example ASC/Caspase 1/IL-1β/IL-18).
NLRs are intracellular proteins containing leucine-rich repeats (LRRs) and an effector binding domain for example PYD. After binding of the adaptor molecule ASC by PYD/PYD interaction, the inflammatory pro-caspase 1 is recruited. Autocatalytic cleavage of pro-caspase 1 leads to activation of caspase 1 which than cleaves the pre-forms of IL-1β and IL-18 into the active forms.

1.1.4. Processing and function of IL-1β, IL-18 and IL-33

The cytokines IL-1β, IL-18 and IL-33 expressed in DCs and many other cells are crucial mediators of inflammation and involved in cell recruitment, proliferation, activation, differentiation and apoptosis (Dinarello 2000a; Dinarello 2000b; Dinarello 2009; Martin 2016). They belong to the IL-1 family and play major roles in a wide range of inflammatory, infectious and autoimmune diseases (Dinarello 1996; Dinarello 2005b; Dinarello 2009).

Briefly, IL-1β induces fever, activates other cells to produce inflammatory cytokines and chemokines and induces endothelial cells to express cell membrane adhesion molecules (Romero et al. 1997). It assists in the polarization of human Th-17 cells (Lasiglie et al. 2011; Meng et al. 2009), regulates synaptic plasticity and memory processes and participates in pain development (del et al. 2012; Gabay et al. 2011; Schneider et al. 1998; Yirmiya and Goshen 2011). The gene expression of IL-1β is regulated by the transcription factor NF-κB and the MAP kinases p38 and ERK1(Bauernfeind et al. 2009; Ghonime et al. 2014; He et al. 2012; Song et al. 2007; Tschopp et al. 2003). IL-1β synthesized as inactive pre-form is cleaved upon certain stimulation by the inflammasome (Figure 4) and subsequently released. To a certain extent, each step is regulated independently (Dinarello 2005a; Dinarello 2005b; Jin and Flavell 2010; Man and Kanneganti 2015). Presumably the mechanism for cleavage and activation of IL-18 is identical to IL-1β. Extracellular ATP is one potent stimulus for the IL-1β processing and release by the purinergic plasma membrane receptor $P2X_7$ (Ferrari et al. 2006). In 2001, MacKenzie et al. showed that activation of the $P2X_7$ receptor in macrophages leads to translocation of IL-1β loaded microvesicles towards the plasma membrane (MacKenzie et al. 2001) independent of caspase-1 (Qu et al. 2009). In human monocytes the pro-IL-1β storage and processing probably take place in secretory vesicles, a release of IL-1β upon ATP stimulation is detectable (Andrei et al. 2004). In human DCs the release of IL-1β in microvesicles can also be provoked by stimulation with T-cells or bacterial substances, the maturation status and the ion concentration especially potassium within the media are critical for this process (Pizzirani et al. 2007) (Figure 4). Until now, location and time for packaging of the cytokine into the vesicle remain unknown. Due to the fact that IL-1β and other components of the inflammasome (e.g. activated

caspases) are released together, packaging and cleavage are probably chronologically and spatially coupled. IL-1β exhibits no signal-sequence for the classical secretion pathway across the endoplasmatic reticulum (Dinarello and Margolis 1995) and is released via other mechanisms. In 2012, Dubyak summarized five different secretion pathways, namely exosome pathway, microvesicle pathway, autophagy pathway, secretory lysosome pathway and pyroptotic / necroptotic pathway for the non-classical IL-1β secretion (Dubyak 2012). Using novel real-time single-cell imaging technology in macrophages, IL-1β was secreted only via the pyroptotic / necroptotic pathway by dying macrophages, which can be different in DCs (Liu et al. 2014; Shirasaki et al. 2014).

The release of IL-1β by DCs plays *inter alia* a role in activation of T-cells (Nagelkerken and van Breda Vriesman 1986; Rasanen et al. 1986) and a defective control of it may cause serious diseases.

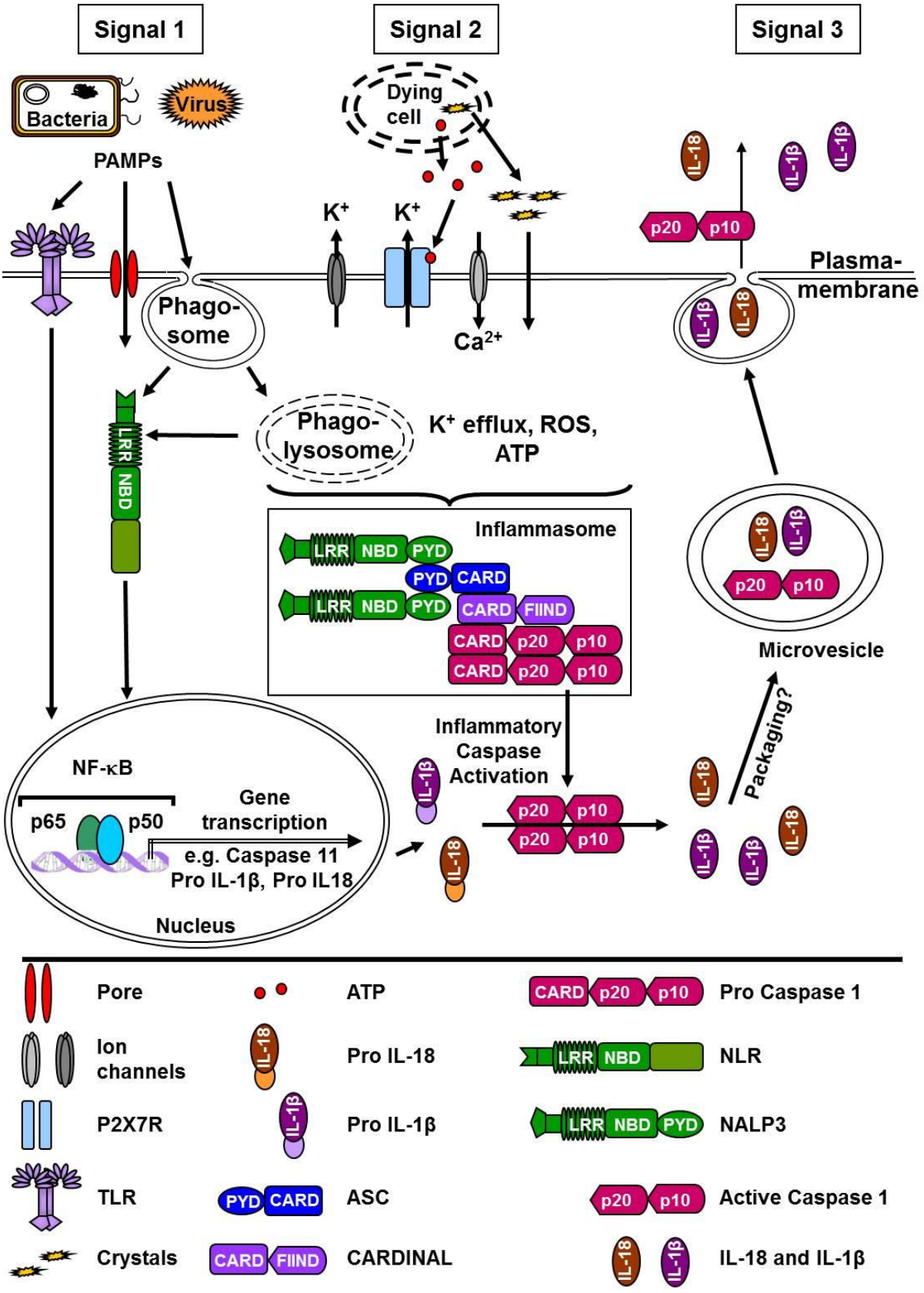

Signal 1
Signal 2
Signal 3
Bacteria
Virus
PAMPs
Dying cell
K+
K+
Ca2+
Plasma-membrane
Phago-some
Phago-lysosome
K+ efflux, ROS, ATP
LRR
NBD
Inflammasome
PYD
CARD
FIIND
p20
p10
IL-18
IL-1β
Microvesicle
Packaging?
Inflammatory Caspase Activation
NF-κB
p65
p50
Gene transcription
e.g. Caspase 11
Pro IL-1β, Pro IL18
Nucleus
Pore
Ion channels
P2X7R
TLR
Crystals
ATP
Pro IL-18
Pro IL-1β
ASC
CARDINAL
Pro Caspase 1
NLR
NALP3
Active Caspase 1
IL-18 and IL-1β

Figure 4

Figure 4: Synthesis, activation and secretion of IL-1β.
PAMPs of bacteria and viruses recognized by TLRs or NLRs (micropinocytosed, phagocytosed or directly bound) lead to gene transcription and cytokine preform synthesis by activation of NF-κB. Recognition of PAMPs intracellularly by NLRs results in inflammasome complex formation which is also influenced / induced by changed ion concentration due to dying cells, perturbation of mitochondrial function and extracellular ATP. Caspase activation within the inflammasome induces cleavage and maturation of e.g. IL-1β and IL-18. One non-classical secretion pathway for the cytokines, namely the microvesicle pathway, is illustrated.

The cytokine IL-18 is expressed as pre-form in DCs, macrophages and epithelial cells. It can be cleaved by caspase-1 (Gu et al. 1995; Kuida et al. 1995), by proteinase-3 (Sugawara et al. 2001) or by serin proteases like cathepsin-B (Hentze et al. 2003). Additionally cleavage by caspase-3 into biologically inactive peptides was described (Akita et al. 1997). Furthermore, the molecular background of cleavage and release of IL-18 *in vitro* and *in vivo* seems to be similar to IL-1β. IL-18 leads to Th1 or Th2 polarization depending on the way and context of stimuli, cellular properties, ambient cytokine milieu and genetic impact (Dinarello 2000b; Okamura et al. 1995).

The cytokine IL-33 (IL-1F11) mediates biological effects by IL-1 receptor ST2, activates NF-κB and MAP kinases in mast cells leading to the production of Th2-associated cytokines of *in vitro* polarized Th2 cells and increased IgE and IgA levels. It induces *in vivo* the expression of IL-4, IL-5 and IL-13 (Schmitz et al. 2005). On the mRNA level, it is expressed in many tissues like lymph nodes, stomach, lung, spleen, bone marrow, brain, skin, pancreas, kidney and heart (Schmitz et al. 2005). Interestingly, it is expressed in Th2 cells, bone marrow derived LPS activated macrophages and bone marrow derived non-LPS activated DCs. The protein IL-33 is constitutively expressed in high amounts in the nucleus of endothelial cells in most human tissues (Kuchler et al. 2008; Moussion et al. 2008). It is synthesized as biologically active full-length protein and can be cleaved by caspase-1, caspase-3 or neutrophil proteinase 3 (Bae et al. 2012). In 2009, Cayrol and Girard proposed that IL-33 is a dual-function protein playing important roles as cytokine and intracellular nuclear factor, like shown for IL-1α and the chromatin associated cytokine HMGB1 (Agresti and Bianchi 2003; Carriere et al. 2007; Cayrol and Girard 2009; Roussel et al. 2008; Scaffidi et al. 2002; Wang et al. 1999a; Werman et al. 2004). Full-length IL-

33 can be released in the extracellular space after endothelial cell damage or injury. Secreted IL-33 signals through the TLR/IL-1R/MyD88 signalling pathway (Ho et al. 2007; Kondo et al. 2008; Schmitz et al. 2005) and probably acts like IL-1α as endogenous DAMP (Bianchi 2007). IL-33 seems to be a promising therapeutic target for several diseases including asthma, rheumatoid arthritis, atherosclerosis and cardiovascular diseases (Carriere et al. 2007; Kakkar and Lee 2008; Miller et al. 2008; Sanada et al. 2007; Schmitz et al. 2005; Xu et al. 2008).

1.1.5. Inhibition of the inflammasome by pathogens

Specific mechanisms for regulation, modulation and activation of inflammasomes by endogenous host systems (inflammatory response regulation) and by microbial pathogens as survival and defense strategy are still in the nascent stages of characterisation. Inflammasomes are stringently regulated by intracellular ion concentrations and potassium is one key player (Khare et al. 2014; Latz et al. 2013; Munoz-Planillo et al. 2013).

As one defense strategy, viral pathogens evolved virulence factors like viroporins, which may alter host cell membrane permeability and the ionic milieu of cell membranes to antagonize inflammasome pathways (Chen et al. 2007; Guo et al. 2015a; Guo et al. 2015b).

Secondly, cellular or host-encoded molecules directly target proteins in the inflammasome and contain one single death fold domain, which functions as dominant- negative inhibitor. They can be distinguished into two classes of proteins the PYD-only proteins (POPs) (Bedoya et al. 2007; Chae et al. 2003; Chae et al. 2006; Stehlik et al. 2003; Yu et al. 2006), of which approximately 20 members have been identified in human, and the CARD-only proteins (COPs) (Druilhe et al. 2001; Humke et al. 2000) (Table 1). Some Gram-positive and Gram-negative virulence factors of bacteria can interact directly and indirectly with inflammasomes and modulate for example caspase-1 function (Schotte et al. 2004). Poxvirus-encoded PYD proteins like M13L-PYD display an anti-inflammatory strategy by interaction with ASC and thus inhibition of caspase-1 activity (Johnston et al. 2005) (Table 1). Other viral POPs not mentioned in table 1 have also been identified in various viruses

including Tanapox, Yaba-like disease virus, suipoxvirus swinepoxvirus and leporipoxvirus Shope fibroma virus (Schotte et al. 2004).

Thirdly, the regulation of host gene expression including post-transcriptional regulators such as micro RNAs to control cellular factors involved in defense mechanisms of the host is used extensively by viruses to propagate their progeny (Gan et al. 2015; Tycowski et al. 2015; Yang et al. 2014).

Fourthly, some viruses express multifunctional proteins, which inhibit e.g. the inflammasome assembly (Gerlic et al. 2013; Lupfer and Kanneganti 2012; Marshall et al. 2015) or directly caspase-1 like for example CrmA (Kettle et al. 1997; Petit et al. 1996; Ray et al. 1992; Stasakova et al. 2005).

All the strategies developed evolutionary by several viruses enable them to establish a productive infection and to adapt and evade the host immune response (Devasthanam 2014; Gram et al. 2012).

Table 1 Cellular and microbial inhibitors of inflammasome activity (adapted from: (Johnston et al. 2007; Stehlik and Dorfleutner 2007)
cPOP: cellular PYD only protein
Yop: Yersinia encoded YOP (Yersinia outer proteins) effector proteins
M13L: prototypical poxvirus-encoded POP from the leporipoxvirus, myxoma virus

Source	Protein	Target(s)	Proposed effect
Host cell	Pyrin	ASC	Blocks inflammasome assembly
		Caspase-1	Directly blocks caspase activation
	cPOP-1	ASC	Inhibits inflammasome assembly and NF-κB activation
	cPOP-2	PYD-NLRs	Inhibits inflammasome assembly and NF-κB activation
	COP	Caspase-1	Blocks caspase recruitment
	ICEBERG	Caspase-1	Blocks caspase recruitment
Virus	M13L	ASC	Blocks inflammasome assembly
Bacteria	YopE	Caspase-1	Blocks caspase autocleavage
	YopT	Caspase-1	Blocks caspase autocleavage
	YopP	NF-κB	Blocks NF-κB signalling

1.1.6. The role of the inflammasome in DCs

Various biological activities such as inflammation, innate immunity, immune cell proliferation, vascularization, wound healing, immune cell differentiation and adaptive immunity are induced by inflammasome-activated IL-1 family ligands (Kwak et al. 2016). Briefly, DCs are activated in close proximity of an infection; recognized PAMPs and DAMPs lead to the formation and activation of the inflammasome. The activation of the inflammasome induces cytokine processing, e.g. IL-1β, important for activation of innate and adaptive immune responses (Ogura et al. 2006). These inflammatory cytokines are released by the cell into the environment; other immune cells are recruited to the place of infection to eliminate pathogens. Upon activation and induction of maximal immune response, DC cell death is initialized by intrinsic, extrinsic and T-cell mediated signals (Kushwah and Hu 2010). The cell death seen upon inflammasome activation is a form of a programmed necrotic cell death called pyroptosis (Jorgensen and Miao 2015). Pyroptosis depends on the activation of at least one inflammatory caspase such as caspase-1 and caspase-11 in mice, and caspase-1, -4 and -5 in humans (Cerretti et al. 1992; Thornberry et al. 1992). The initiated caspase activation upon inflammasome activation results in the formation of pores by the N-terminal cleavage product of gasdermin D in the plasma membrane and finally to the lysis of the cell (Fink and Cookson 2006; Kayagaki et al. 2011; Vince and Silke 2016). A robust inflammatory response is induced by the rapid plasma membrane rupture and the release of DAMPs and cytokines (Jorgensen and Miao 2015). Upon inflammasome activation, released ATP, which activates $P2X_7$, is one key autocrine signal to induce cytotoxicity and pyroptosis (Chekeni et al. 2010; Yang et al. 2015). The actin cytoskeleton gets destroyed in pyroptotic cells (Denes et al. 2012; Lamkanfi and Dixit 2014; Miao et al. 2011); DNA gets fragmented and nuclear condensation takes place (Asgari et al. 2013; Lopez-Castejon and Brough 2011). The removal of infected cells further stimulates the activation of the immune system via the release of pathogens from dying cells. So, the inflammasome plays an important role not only for activation of the immune response but also for clearance of infected cells from an organism (Aachoui et al. 2013; Casson and Shin 2013). Furthermore, the uptake of apoptotic DCs by viable DCs can also induce tolerance by priming of antigen specific regulatory T cells (Kushwah et al. 2009; Kushwah et al. 2010; Oh and Shin 2015).

A diminished immune response after circumvention and/or blocking of the inflammasome by bacteria or viruses can lead to severe infections by uncontrolled pathogen proliferation in the infected cells including infection of new cells. So clearance of infected cells by apoptosis and/or pyroptosis is an important factor during immune reactions of an organism.

1.2. Role of cell death for regulation of DC immune response

1.2.1. The different faces of cell death

Cell death is essential for cell homeostasis and the development and maintenance of multicellular organisms (Trosko and Ruch 1998). Classified according to its appearance, there are two well-known forms of cell death: apoptosis and necrosis (Kroemer et al. 2009).

Necrosis, induced by injury, infection, inflammation or toxins, is characterized by gain in cell volume, swelling of organelles and a release of intracellular content after cellular membrane damage leading to inflammation (Festjens et al. 2006; Haslett 1992; Kroemer et al. 2009). As a form of the so called caspase-independent cell death (CICD), necrosis often shares common characteristics with apoptotic cell death (Tait and Green 2008) such as upstream signalling pathways like mitochondrial membrane permeabilization. Cells undergoing CICD are characterized by large-scale cytoplasmic vacuolization, autophagosome accumulation and peripheral nuclear condensation (Ekert et al. 2004). One special form of necrotic cell death is the caspase-dependent pyroptosis (Assuncao and Linden 2004). Pyroptosis as an inflammatory form of a programmed cell death is essential for controlling microbial pathogen infections. Depending on inflammasome activation and resulting in the release of pro-inflammatory cytokines like IL-1β and IL-18, pyroptosis occurs in macrophages, monocytes and dendritic cells and leads finally to cell death (Zhang et al. 2018). In the early stage of pyroptosis, a large number of vesicles called pyroptotic bodies are produced leading to pores and finally to a rupture of the cell membrane (Chen et al. 2016).

Apoptosis, a programmed suicide, can occur e.g. if life span of a cell is over or if the cell was damaged by mechanical, chemical, physical or biological influences (Hanawalt 1996; Sinkovics 1991). It is morphologically characterized by a reduction of the cellular volume, condensation of chromatin, nuclear DNA fragmentation, membrane blebbing and constriction of apoptotic bodies (Kroemer et al. 2009; Wyllie 1997). Apoptosis is a caspase-dependent cell death (CDCD) initiated by activating the death program of a cell. Apoptosis does not lead to an inflammation reaction, cell fragments stay enveloped and are quickly digested by macrophages and neighbouring cells (Kerr et al. 1972). The time lapse of apoptosis can be divided into three successive steps: induction- , regulation- and termination phase.

There are two distinct ways for induction of apoptosis, one extrinsic pathway e.g. by stimulation of certain surface receptors like death receptors and one intrinsic, where the mitochondria play a central role (Hengartner 2000; Roy and Nicholson 2000).

1.2.2. The intrinsic cell death pathway

Various circumstances, like growth factor withdrawal or DNA damage by oxygen radicals, radiation and chemotherapeutics activate the intrinsic signal transduction (mitochondrial pathway) (Hotchkiss et al. 2002). The mitochondria as common damage sensors release mitochondrial factors like cytochrom c, which initiates a macromolecular complex called apoptosome (protein complex consisting of caspase-9, AIF and cytochrome c) and thereby activates procaspase-9 (Green 2000; Green and Beere 2000; Green and Llambi 2015). Apoptosome formation leads to activation of executioner caspases like caspase-3, which targets and inhibits other downstream pro-survival molecules (Beere 2005). Chromatin condensation and DNA fragmentation finally characterizes this type of cell death (Beere 2005). Biochemical key players in the intrinsic pathway are proteins of the BCL-2 family consisting of both anti- and pro-apoptotic members. BH3-only proteins like BID, BAD, NOXA, PUMA, BIM and BMF act upstream in the signalling cascade and detect developmental death signals or intracellular damage (Festjens et al. 2006; Sasi et al. 2009). BAX and BAK as pro-apoptotic members act downstream in the pathway mediating mitochondrial disruption (Cory et al. 2003; Sasi et al. 2009).

Inhibition of BAK e.g. plays an important role for the survival of DCs during differentiation and activation (Lehner et al. 2012). The anti-apoptotic members BCL-2 and BCL-X_L are involved in maintaining the integrity of the mitochondrial outer membrane, endoplasmatic reticulum and nucleus and may function as oncogenes (Pattingre and Levine 2006). Modulation of pro- and anti-apoptotic Bcl-2 family proteins by extracellular (also microbial) and intracellular signals is critical for cell survival and their balance decides over the fate of cell.

1.2.3. CD95L-mediated apoptosis as an extrinsic cell death pathway

The extrinsic way is characterized by signal transduction from extracellular death ligands most of them belong to the TNF-superfamily like TNF and CD95 Ligand (CD95L). Binding of the extracellular ligand to its receptor leads to apoptotic cell death. Beside the TNF receptors like TNF-R1, also CD95 (Fas-receptor), the TRAIL receptors TRAIL-R1 (death receptor 4 (DR4)), TRAIL-R2 (DR5), TRAIL-R3 and TRAIL-R4, DR3 and DR6 belong to the TNF-receptor family (Ashkenazi and Dixit 1998; Leverkus et al. 2000a; Locksley et al. 2001; Wachter et al. 2004; Walczak et al. 1999).

The most studied apoptosis stimulus is the apoptosis induction by CD95-CD95L-interaction (Krammer 2000). CD95L, also known as FasLigand, is a type-2 transmembrane protein of 40 kDa (Nagata 2000). Its expression is strongly regulated and was detected on activated T lymphocytes and NK-cells, vascular endothelium and so-called immune privileged organs like brain, eye, ovary and testicle (Newell and Desbarats 1999). The receptor for CD95L is CD95, a type-1 transmembrane protein of 45 kDa (Figure 6) (Walczak and Krammer 2000).

CD95 is ubiquitarly expressed, but is particular strongly expressed on thymocytes, activated T- and B-lymphocytes, hepatocytes, heart and kidney cells (Nagata 2000). Alternative splice variants of CD95 without transmembrane domain are soluble (Cascino et al. 1995) and can bind and inactivate CD95L (Papoff et al. 1996).

Briefly, the signal transduction starts with binding of CD95L to the receptor CD95 leading to trimerization (Schulze-Osthoff et al. 1998) and recruitment of the adaptor molecule Fas-associated death domain (FADD) by interaction of the death domains (DDs) respectively. FADD contains a death effector domain (DED) to which e.g. the inactive (initiator) pro-caspase-8 can bind (Cohen 1997), thus building the death-inducing signalling complex (DISC) containing FADD, the initiator caspases-8 and/or -10, and/or the caspase-8 homolog cFLIP, RIPK1 and/or other potential unknown molecules (Figure 5) (Kischkel et al. 1995; Peter 2004; Riedl and Salvesen 2007). Active caspase-8 is formed by an autoproteolytic cleavage of pro-caspase-8 (Medema et al. 1997; Siegel et al. 2000). Caspase-8 activates effector caspases such as caspase-3 or the Bcl-2 protein Bid that trigger the activation of the mitochondrial apoptotic signalling pathway. These secondarily activated caspases induce the detectable morphological and functional modifications in apoptotic cells. The proteins of the cytoskeleton like actin (Mashima et al. 1997), gelsolin (Kamada et al. 1998) and α-fodrin (Martin et al. 1996) beside other various cellular proteins are important substrates of those effector caspases.

Caspase-dependent desoxyribonuclease (CAD) cleaves the DNA between the nucleosome in fragments of about 180 base pairs (Sakahira et al. 1998). This represents the end of nuclear apoptosis characterized by compact condensation of the chromatin with or without building of apoptotic bodies (Daugas et al. 2000; Loeffler et al. 2001).

Members of the death receptor family regulating apoptosis signalling cascades are of huge importance for the immune system, disruption of the pathways leads to development of autoimmunity in various organ systems (Locksley et al. 2001; Rieux-Laucat 2006; Walczak and Krammer 2000; Wang et al. 1999b).

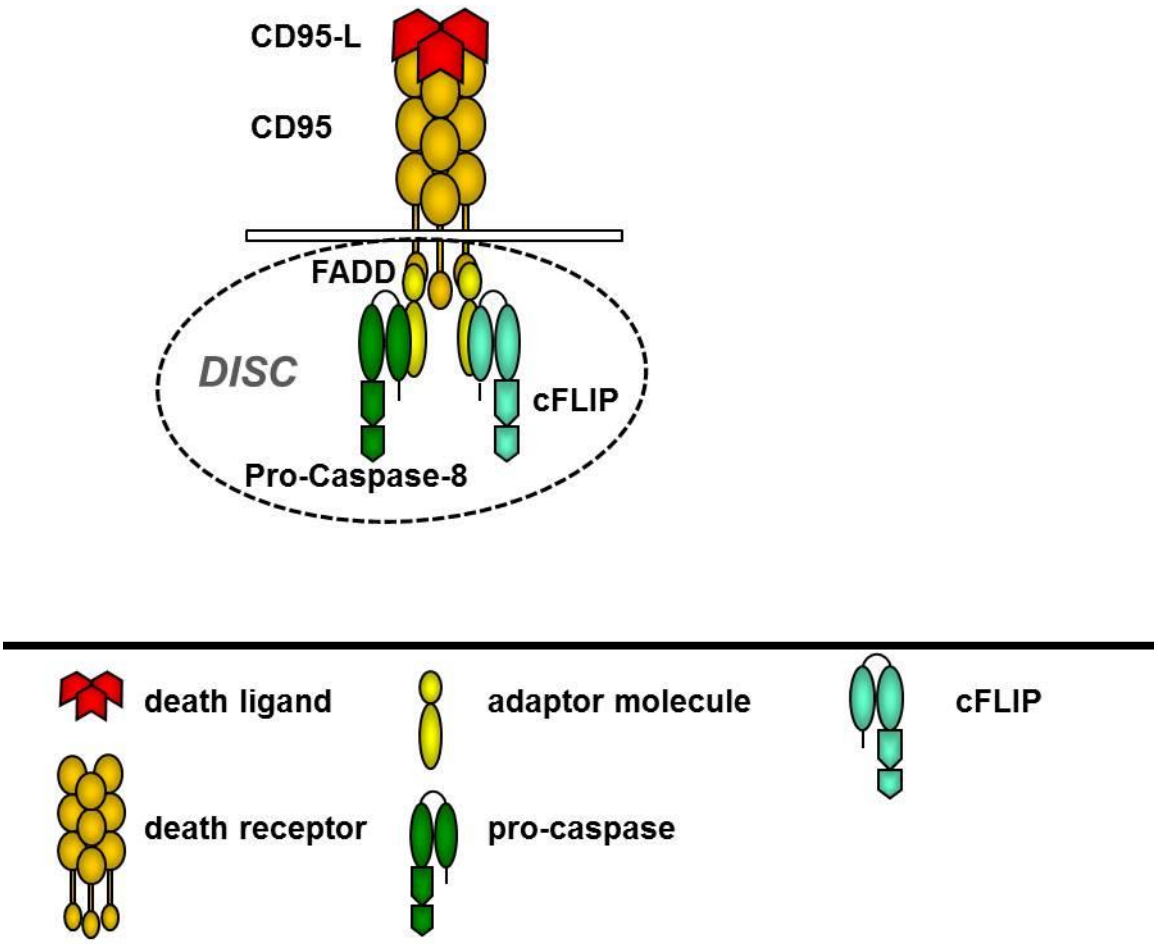


Figure 5

Figure 5: Formation of membrane-bound CD95 death receptor complex.
Activation of death receptor triggers recruitment of different receptor associated molecules to the receptor. Formation of a membrane bound receptor complex occurs (Geserick et al. 2009).

1.2.4. Inhibition of CD95-mediated apoptosis by cFLIP

Apoptotic signals are regulated at different levels beyond activation of effector caspases. The modification of the density of cell surface death receptors on expression level influences the sensitivity of the cell against death ligand-induced apoptosis. Upon endogen stimuli like for example growth factor deprivation, DNA damage and cellular stress, proteins of the Bcl-2 family decide about apoptosis on the mitochondrial level (Green 2000; Youle and Strasser 2008).

Furthermore, processed caspases can be blocked by intracellular inhibitors of apoptosis, the so called IAPs (Inhibitors of Apoptosis Proteins) (Feoktistova et al. 2011; Geserick et al. 2009; Huang et al. 2000; Liston et al. 2003; Salvesen and Duckett 2002; Yang and Li 2000). IAPs act as endogenous regulators of caspases (Fan et al. 2005). These can inhibit several caspases by promoting their degradation or by binding and sequestering of these caspases (Tenev et al. 2005).

In 1997 a viral protein containing two DEDs was described which blocks effectively apoptosis upon stimulation of death receptors (Bertin et al. 1997; Hu et al. 1997b; Thome et al. 1997). It was shown that this protein, called vFLIP, inhibits the recruitment of procaspase8/FLICE to the receptor signalling complex. Subsequently, cFLIP was described as relative to the viral protein vFLIP (Goltsev et al. 1997; Han et al. 1997; Hu et al. 1997a; Inohara et al. 1997; Irmler et al. 1997; Shu et al. 1997; Srinivasula et al. 1997). Until now, more than 11 different splice variants are known on mRNA level, at least three of them expressed as protein: $cFLIP_S$ (26 kDa), $cFLIP_R$ (24 kDa) and the 55 kDa variant $cFLIP_L$ (Djerbi et al. 2007; Golks et al. 2005; Scaffidi et al. 1999c).

cFLIP is substantially and constitutively expressed in numerous cells including neurons, myocytes, endothelial cells, keratinocytes, β-cells, DCs, macrophages, $CD34^+$ hematopoetic stem cells and spermatocytes (Armbruster et al. 2009; Cottet et al. 2002; Dutton et al. 2004; Giampietri et al. 2003; Huang et al. 2010; Imanishi et al. 2000; Irmler et al. 1997; Kim et al. 2002; Leverkus et al. 2000b; Scaffidi et al. 1999a). cFLIP expression is controlled by different signalling pathways like for example ERK, CREB, TGFβ and in part AKT (Micheau et al. 2001; Panka et al. 2001; Schlapbach et al. 2000; Wang et al. 2002; Wang et al. 2008; Zhang et al. 2008). It can be upregulated by TNF, IL-1, LPS or CD40 (Aggarwal et al. 2004; Budd et al. 2006; Franchi et al. 2003; Leverkus et al. 2000b; Sakuraba et al. 2007; Takahashi et al. 2006; Wachter et al. 2004). Beside the evidence of 11 distinct isoforms of the cFLIP gene by alternative splicing, only a long ($cFLIP_L$) and a short ($cFLIP_S$) isoform are detected in most human cells (Budd et al. 2006; Kataoka 2005; Scaffidi et al. 1999c). In 2008, Ueffing N. et al. showed, that $cFLIP_R$ is the only short isoform expressed in mice (Ueffing et al. 2008).

The proteins vFLIP (Thome et al. 1997), $cFLIP_R$, $cFLIP_S$ and $cFLIP_L$ protect cells against receptor-mediated apoptosis, induced by different death receptors like CD95, TNFR1, TRAILR1 (DR4), TRAILR5 (DR5) and DR3 (Condorelli et al. 1999; Golks et al. 2006; Hu et al. 1997a; Irmler et al. 1997; Rasper et al. 1998; Scaffidi et al. 1999c; Srinivasula et al. 1997).

Inhibition of apoptosis results from the recruitment of cFLIP molecules to the DISC (Budd et al. 2006; Krueger et al. 2001; Scaffidi et al. 1999b). The different mode of inhibition is caused by the differences of the splice variants (Figure 6).

$cFLIP_L$ is structurally similar to Caspase-8 and Caspase-10, but the C-terminal part is lacking enzymatic activity (Figure 6). $cFLIP_S$ can fully block the autocatalytic cleavage of caspase-8 bound to the receptor complex whereas $cFLIP_L$ accelerates the autocatalytic cleavage of caspase-8 to the p43/41 fragment, but blocks further cleavage thereby fixing the enzymatic active p43/41 fragment in the receptor complex (Geserick et al. 2008; Krueger et al. 2001; Micheau et al. 2002).

Upon heterodimerization of Procaspase 8 and cFLIP molecules ($cFLIP_L$ and $cFLIP_S$) cleavage of a 22 kDa N-terminal fragment of cFLIP (p22-FLIP) has been reported (Golks et al. 2006). In this context a classical autoproteolytic cleavage of procaspase was not found. The p22-FLIP-molecule is recruited to the CD95 receptor complex upon receptor stimulation and inhibits efficiently CD95L-mediated apoptosis (Golks et al. 2006). Furthermore, p22-FLIP strongly activates NF-κB in contrast to the p43 cleavage product of cFLIP inhibiting e.g. TRAIL-induced NFκB activation in keratinocytes (Desbarats et al. 2003; Kreuz et al. 2004; Wachter et al. 2004; Wajant et al. 2003).

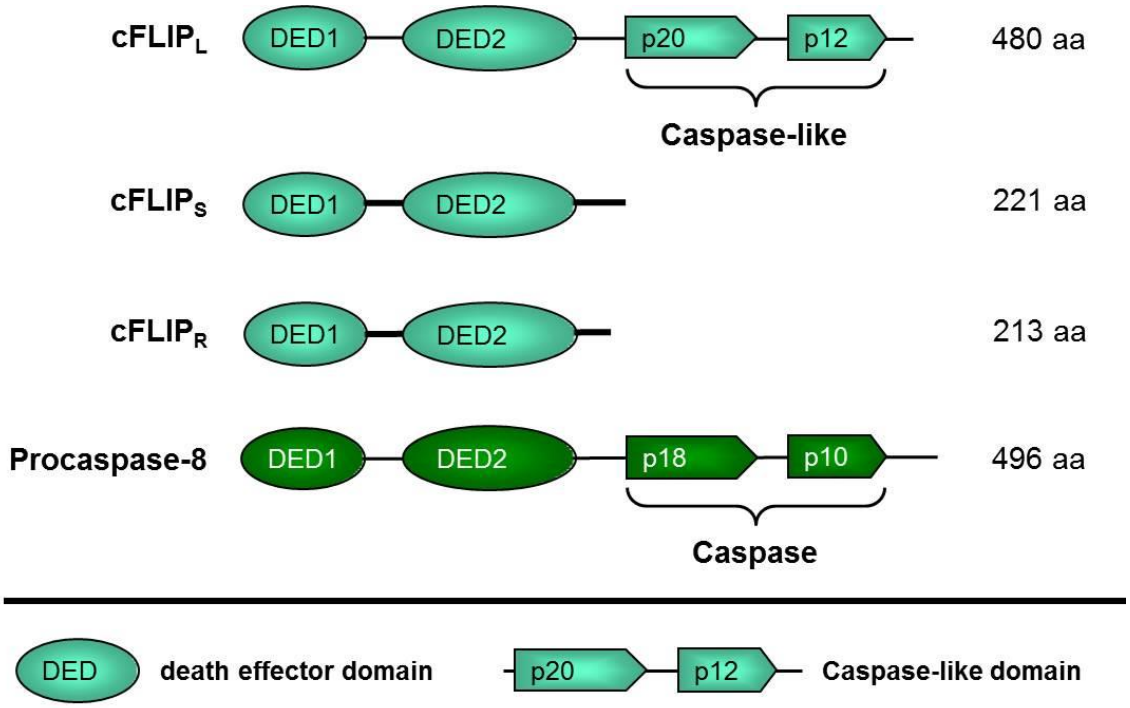


Figure 6

Figure 6: Structures of cFLIP$_L$, cFLIP$_S$, cFLIP$_R$, FADD and procaspase-8. Scheme of domain composition of cFLIP isoforms, FADD and the procaspase-8 molecule is illustrated. All cFLIP isoforms processes two DEDs at the N-terminus, required for interaction with FADD. cFLIP$_L$ contains in addition a caspase-like domain, which is highly homologous to the caspase domain of procaspase-8. Number of amino acids (aa) indicate the lengths of proteins (Geserick et al. 2008; Krueger et al. 2001).

1.2.5. Apoptosis in dendritic cells

Dendritic cell apoptosis attach great importance in regulation of the equilibrium between tolerance and immunity through many different pathways (Kushwah and Hu 2010; Wong et al. 1997). Thereby, the magnitude of an immune response by limiting the antigen (Ag) availability to TCs by extrinsic and TC-mediated signals is regulated. Defects in DCs apoptosis have been associated with different pathologies like sepsis, breast cancer and autoimmune diseases (Chen et al. 2006c; Ito et al. 2006; Pinzon-Charry et al. 2007).

DC life span is fairly limited (Kamath et al. 2000); DC apoptosis is multifaceted as multiple pathways are involved.

Three divergent ways of receptor-mediated apoptotic cell death are described in DCs. In immature DCs and their precursor cells, the classical caspase-dependent signalling pathway via death receptors like CD95 and TRAIL-R leads to apoptosis (Budd 2002; Leverkus et al. 2000b; Willems et al. 2000). Mature DCs can die by an MHC class II molecule mediated process; known TC-dependent signals for DC apoptosis are TRANCE, CD154 and CD95L (Chino et al. 2009; Riol-Blanco et al. 2009; Willems et al. 2000; Wong et al. 1997), whereas amyloid peptides, TRAIL, LPS, Type I IFN, Leptin and CCR7 are TC-independent signals inducing DC death (Blum et al. 2006; Escribano et al. 2009; Mattei et al. 2009; Mattioli et al. 2009). In mature DCs, LPS induces apoptosis independent of the TLR4 signalling cascade through CD14 mediated NFAT activation, which is also required during DC maturation processes (Zanoni et al. 2009). Furthermore, mitogen-inducible nuclear orphan receptor (*MINOR*) has been identified to induce DC apoptosis by an unknown signal (Wang et al. 2009).

A third signalling pathway dependent on inflammasome activation in DCs is mediated by the purinergic receptor $P2X_7$ (Coutinho-Silva et al. 1999) probably identical to a process described as pseudo-apoptosis (Mackenzie et al. 2005) later known as pyroptosis dependent on the inflammasome activation is DCs (Jorgensen and Miao 2015). Additionally, caspase-dependent and caspase-independent mechanisms upon DC-TC interaction were described without further characterisation of the signalling pathways (Kitajima et al. 1996). Apoptosis and caspase-dependent as well as caspase-independent processes in mature DCs probably play a role in termination of immune responses therefore regulating self-tolerance and preventing autoimmune diseases (Chen et al. 2006b; Chen et al. 2006c).

In the year 2000, Leverkus et al. showed that during maturation of DCs the upregulation of the caspase-8 homolog cFLIP leads to circumvention of CD95L-induced apoptosis in mature DCs without changing CD95 expression (Leverkus et al. 2000b). In this regard, Lehner et al. demonstrated in 2012 by knockdown experiments using small interfering RNAs that cFLIPL as well as BAK and Bcl-2 is regulated by autocrine TNF, a critical factor for the survival of human DCs (Lehner et al. 2012; Leverkus et al. 2003a). In murine DCs the sensitivity to CD95L correlates with maturation status as well (Leverkus et al. 2000b; McLellan et al. 2000). Furthermore, initiation of the CD95 signalling cascade in DCs can lead to maturation

and influences the immune response via a non-apoptotic CD95-mediated signalling pathway (Franchi et al. 2003; Guo et al. 2005; Rescigno et al. 2000), which is probably modulated by signals (e.g. CD40L, TRANCE) activated during DC-TC interaction (Mehling et al. 2001; Wong et al. 1997).

In human DCs, the p22-FLIP fragment can also be found upon maturation, perhaps mediating NFκB activation (Golks et al. 2006). Otherwise the p43-fragment is found in mature DCs upon stimulation of death receptors (especially CD95) without provoking apoptosis (Kim et al. 2008). Various stoichiometries and non-identified cofactors could be relevant for the different signalling pathways. Effects of diverse cFLIP cleavage products on non-apoptotic signalling pathways like NF-κB activation in DCs need further investigation.

1.3. Aims

Dendritic cells are an attractive potential therapeutic target for cancer immunotherapy (Banchereau and Steinman 1998; Steinman 1991), playing a key role in the initiation of immune responses. Upon activation, DCs adopt characteristic gene expression pattern relevant for optimal immune responses and concomitant triggering of cell death signalling. This work focuses on elucidating some of the key roles of DCs in their contribution to inflammasome function and CD95L signalling. A lentiviral transduction system of the third generation, developed in 1998 from Dull T et al. and utilized by Oki et al. in 2001 for genetic manipulation of $CD34^+$ precursor cells was established (Masurier et al. 2007) to illuminate and to interfere with inflammasome and death receptor signalling pathways in DCs (Dull et al. 1998; Masurier et al. 2007; Oki et al. 2001).

During this thesis, the following questions were addressed:

1. Does cytokine expression and IL-1β secretion correlate with the maturation status of DCs over time, and on which parameters does it depend on? Especially the role of the $P2X_7$ receptor in DCs is investigated, since it was reported that stimulation of the $P2X_7$ receptor enhances cytokine secretion in monocytes and macrophages (Ferrari et al. 1997; Ferrari et al. 2006; Piccini et al. 2008).
2. Does the expression of a POP like dnASC block the inflammatory complex formation and what is its impact on the IL-1β secretion in DCs?
3. What is the impact of inflammatory cytokines, especially IL-1β, on TC priming and their role in the $CD4^+$ TC-DC *in vitro* interaction model system? Especially the cytokine panel after this interaction is investigated to elucidate the direction of immune response and TC differentiation (Watanabe et al. 2008).
4. Stringent cell death regulation during DCs life span is important for the immune response. So, how does especially CD95L-induced death signalling molecule expression pattern look like in murine DC model in respect to their maturation status? Furthermore, the role of caspases and RIP-1 kinase is investigated for dissection of the observed CD95L-induced cell death.

5. Is a death inducing signalling complex (DISC) formed in DCs (mature and immature) upon stimulation with CD95L like shown in stimulated skin tumor cells (Feoktistova et al. 2011; Geserick et al. 2009), and how does it look like?
6. How does a genetic deletion of cFLIP using the transgenic mouse model system bearing the cFLIP gene flanked by loxP sites ($cFLIP^{fl/fl}$) (Yeh et al. 2000) or a lentiviral cFLIP-knockdown (Szulc et al. 2006) influence the CD95L-sensitivity of DCs and their lifespan? Especially the impact on TC priming in the $CD4^+$ TC – cFLIP-knockdown DC *in vitro* interaction model system and the cytokine panel after this interaction is investigated to elucidate the direction of immune response and TC differentiation.

2. Material and Methods

2.1. Material

2.1.1. Chemicals

The kits and standardized chemicals used in this work were purchased from the following companies and the quality of the reagents was of analytical grade. Special reagents used for some experiments are described at the beginning of each section.

Agarose	Invitrogen, Roth
Albumin from chicken egg white (ovalbumin)	Sigma
Ampicillin sodium salt	Roth
Adenosine-tri-phosphate (ATP)	Sigma
Beta-Mercaptoethanol	Merck, Roth, Sigma
Bicine	Sigma
Bis-Tris	Sigma, Fluka
Brilliant Blue R	Sigma
Bromphenolblue	Sigma
Bovine serum albumin (BSA)	Gibco
Calciumchloride dihydrate	Sigma
Chloramphenicol	Sigma
Chloroquin diphosphate	Sigma
Cholera Toxin	Sigma
Cis dichlorobis triphenylphosphine platinum2 (cis-platin)	Sigma
Coomassie Stain	Biorad
p-Coumaric acid	Sigma
Crystal violet	VWR
Cycloheximide	Sigma
Carboxyfluorescein succinimidyl ester (CFSE)	Fluka
Cell proliferation dye (CPD) eFluor670	eBioscience
DEAE-Dextran hydrochloride	Sigma

Deoxycholic acid sodium salt	Sigma, Fluka
Dimethylsulfoxide (DMSO)	Roth
Dithiotreitol	Roth
Doxycyclin	Hexal
Ethidiumbromide	Roth, Sigma
5-Fluorouracil	Fluka
Gelatine from porcine skin typeA	Sigma
Hexadimethinebromide (Polybrene)	Sigma
Histodenz	Sigma
Kanamycin sulphate	Roth
L-Cysteine	Fluka
Lipofectamine	Invitrogen
Luminol	Sigma
Ponceau S	Roth, Sigma
Propidium iodide	Sigma
Protamine sulfat	Sigma
Rubidium chloride	Sigma
Silver nitrate	Sigma, Roth
Stripping Buffer, Restore WesternBlot	Thermo Scientific

2.1.2. Enzymes and molecular biology reagents

Restriction endonucleases type 2, ligases, polymerases and the corresponding 5x and 10x concentrated reaction buffers were purchased from Promega and Fermentas. Subsequent endonucleases were used in this work: *BamH*I, *EcoR*I, *EcoR*V, *Hind*III, *Hpa*I, *Nco*I, *Nhe*I, *Xba*I, *Xho*I.

Alkaline Phosphatase from calf intestine (CIAP)	Promega
Thermosensitive Alkaline Phosphatase (TSAP)	Promega
Deoxynucleoside Triphosphate Set (dNTPs)	Promega, Fermentas
Endonucleases (Restriction enzymes)	Promega, Fermentas
NuPAGE® Novex 4-12% Bis-Tris Gels	Invitrogen
Oligonucleotides (Primer)	MWG (LCC genomics)

Polyvinylidene fluoride (PVDF) membrane	GE Healthcare
Protein G beads	Roche
T4 DNA ligase	Promega
Taq DNA polymerase	Promega, Fermentas
Pfu DNA Polymerase	Promega
DNA ladder (100 bp and 1 kb)	Promega

2.1.3. Ready-made reaction systems (Kits)

ECL Detection Reagents	GE Healthcare
ECL Plus™ Western Blotting Detection Reagents	GE Healthcare
Luminata™ Forte Western HRP Substrate	Millipore
EndoFree Plasmid Maxi Kit	Qiagen
Wizard Plus SV Miniprep DNA Purification System	Promega
Wizard® SV Gel and PCR Clean-Up System	Promega
DC Protein assay	Bio-Rad
ELISA (mouse IL-2, mouse IL-1β, human IL-1β)	eBiosciences
ELISA (mouse IL-18)	MBL (Biozol)
ELISA (mouse IL-33)	R&D Systems
Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex kit	eBiosciences
Pan T Cell Isolation Kit II, mouse	Miltenyi Biotec
Bradford assay	BioRad

2.1.4. Phosphatase and protease inhibitors

AEBSF Hydrochlorid	AppliChem
Aprotinin	Roth
Benzamidine	Fluka
Complete (Protease Inhibitor Cocktail Tablets)	Roche
β-Glycerophosphate disodium salt hydrate	Sigma
Leupeptin hemisulfate salt	Sigma
Sodium orthovanadate	Sigma
Sodium pyrophosphate tetrabasic decahydrate	Sigma

2.1.5. Pharmacological stimulating substances

4-Hydroxytamoxifen (4-HT)	Sigma
z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD-fmk)	Bachem
N-(2-Quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl Ketone (QVD)	Merck
Lipopolysaccharide from E.coli 055:B5 and 026:B6	Sigma
D(+) Mannose	Fluka
2´(3´)-O-(4-Benzoylbenzoyl)adenosine 5´-triphosphate triethylammonium salt (Bz-ATP)	Sigma
Adenosine 5´-triphosphate, periodate oxidized sodium salt (Ox-ATP)	Sigma
Monosodium urate	Alexis, Enzo
Ionomycin	Alexis Biochemicals
Peptidoglycan (PGN)	Sigma
Muramyl dipeptide (MDP)	Bachem
Mannan	Sigma
Phorbol 12-myristate 13-acetate (PMA)	Sigma
Lipoteichonsäure	Sigma
Anaphylatoxin	Bachem
Laminarin	Sigma
Hyaluronsäure	Sigma
Mannose-6-P	Fluka
OVA Peptide (323-339)	AnaSpec Inc., (San Jose, USA)
OVA protein (grade III)	Sigma

2.1.6. Stimulating Cytokines

TNF-R2-Fc	Wyeth Pharmaceuticals
His-Flag-TRAIL (HF-TRAIL)	P. Diessenbacher (Diessenbacher et al., 2008)
CD95L-Fc	M. Feoktistova (Geserick et al. 2009)
TWEAK-Fc	M. Feoktistova (Geserick et al. 2009)
M-CSF	Peprotech
TNF-alpha (mouse)	Immunotools
Prostaglandin-E2	Fluka, Sigma, Axxora

Recombinant murine IL-1β	Strathmann Biotec
Recombinant murine IL-4	Immunotools

2.1.7. Molecular weight markers

GeneRuler™ 1 kb DNA Ladder, ready-to-use	Fermentas
GeneRuler™ 100 bp Plus DNA Ladder, ready-to-use	Fermentas

2.1.8. Protein molecular weight markers

SeeBlue® Pre-Stained Standard	Invitrogen
PageRuler^{TM} Prestained Protein Ladder	Fermentas

2.1.9. Table 2: Primary antibodies for Western blot analysis

Antibody	**Species/Isotype**	**Clone**	**Company**
Mouse Caspase-8	Rat IgG1	1G12	Alexis
Bacteriophage P1 Cre recombinase	Rabbit polyclonal IgG		Novagen
Mouse cFLIP	Rat IgG2a	Dave-2	Apotech / Alexis
Mouse FADD	Rat IgG1 kappa	7A2	Alexis / kindly provided from O'Reilly
β-Actin N-terminal	Rabbit polyclonal IgG		Sigma-Aldrich
β-tubulin	Mouse IgG1		Sigma-Aldrich
Human / mouse Caspase-1*	Mouse IgG1	14F468	Calbiochem
Mouse Caspase-1	Rabbit polyclonal IgG		kindly provided from P. Vandenabele (Van de Craen et al. 1997)
Human / mouse ASC	Rabbit polyclonal IgG	AL177	Alexis
Mouse Caspase-11	Rat IgG	17D9	Sigma
Human / mouse / rat Caspase-12	Mouse IgG1	14F7	MBL
GFP-HRP			Clonetech
Human / mouse IL-1β cleaved	Rabbit polyclonal IgG	D116	Cell signalling
Mouse IL-1β uncleaved	Goat polyclonal IgG	M20	Santa Cruz
Human/mouse/rat/ chicken Ovalbumin	Mouse IgG1	OVA14	Sigma

*The antibody used for Caspase-1 detection was initially developed against full length human Caspase-1 aa 371-390 recognizing mouse Caspase-1 as well and doesn't detect mutual cleavage products. So for detection of murine Caspase-1 and

cleavage products upon activation in Western Blot analysis another antibody developed especially for mouse could give further intracellular details in the signalling cascade. This antibody was not yet available at that time, so experiments were performed with the monoclonal anti-Caspase-1 antibody [14F468].

Western Blot secondary antibodies: Horseradish peroxidase (HRP)-conjugated goat anti-rabbit, goat anti rat IgG, goat anti-mouse IgG. Antibodies and HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG1κ were obtained from Southern Biotechnology Associates.

2.1.10. Table 3: Antibodies for FACS analysis

Antibody	Species/Isotype	Clone	Company
CD86	Rat IgG2a	GL1	eBioscience
CD83	Rat IgG1	Michel-19	Santa Cruz (Biozol)
CD95	Hamster IgG	Jo-2	BD Pharmingen
CD40	Rat IgG2a	FGK45	Own production
CD11c	Hamster IgG	N418	Own production
I-A (b, k)	Mouse IgG2a	K25-137	Own production
CD68	Rat IgG2a	FA-11	AbD Serotec
PE Rat anti-mouse CD4	Rat IgG2a kappa	RM4-5	BD Pharmingen
PE Rat anti-mouse CD8a	Rat IgG2a kappa	53-6.7	BD Pharmingen
PE Hamster Anti-mouse CD11c	Armenian hamster IgG1	HL3	BD Pharmingen
Hamster anti-mouse CD11c	Armenian hamster IgG1	HL3	BD Pharmingen
PE Hamster anti-mouse CD28	Syrian hamster IgG2	37.51	BD Pharmingen
PE Rat anti-mouse CD40	Rat IgG2a kappa	3/23	BD Pharmingen
Rat anti-mouse CD40	Rat IgG2a kappa	3/23	BD Pharmingen
PE Hamster anti-mouse CD80	Armenian hamster IgG2	16-10A1	BD Pharmingen
Anti-mouse CD80 (B7.1)	Armenian hamster IgG2	16-10A1	eBioscience
PE Rat anti-mouse CD86	Rat IgG2a	GL1	BD Pharmingen
Anti-mouse CD86 (B7.2)	Rat IgG2a	GL1	BD Pharmingen
PE rat anti-mouse F4/80	Rat IgG2a	BM8	eBiosciences
Hamster anti-mouse CD95	Armenian hamster IgG2	Jo2	Pharmingen/BD Biosciences
PE anti-mouse CD95L (CD178)	Armenian hamster IgG	MFL3	eBioscience
PE rat IgG2a kappa isotype control	Rat IgG2a kappa	R35-95	BD Pharmingen
PE hamster IgG2 kappa isotype control	Armenian hamster IgG2 kappa	B81-3	BD Pharmingen
PE hamster IgG2 lambda1 isotype control	Armenian hamster IgG2 lambda1	Ha4/8	BD Pharmingen
PE mouse anti-armenian and Syrian hamster IgG cocktail	Mouse IgG1 kappa	G70-204, G94-90.5	BD Pharmingen
PE rat anti-mouse 1-Ad/1-Ed	Rat IgG2a kappa	2G9	BD Pharmingen
PE goat anti-rat Ig	Goat Ig polyclonal		BD Pharmingen
PE mouse anti-rat IgG2a	Mouse IgG2b kappa	RG7/1.30	BD Pharmingen

Rat IgG2b kappa isotype control	Rat IgG2b kappa	RTK4530	Biozol
Rat IgG2a kappa isotype control	Rat IgG2a kappa	RTK2758	Biozol
Rat IgM kappa	Rat IgM kappa	RTK2118	Biozol
Armenian hamster IgG isotype control	Armenian hamster IgG	HTK888	Biozol
Mouse IgG1 control PE-conjugated	Mouse IgG1	203	ImmunoTools

Monoclonal Antibodies for FACScan analysis of surface receptor expression were used as previously described *(Leverkus et al. 2003b).*

2.1.11. Vector backbones

Expression vector: PCR3.1	Invitrogen
pGEM®-T and pGEM®-T Easy Vector Systems	Promega
Lentiviral packaging vector: pMD2.G	(Rubinson et al. 2003)
Lentiviral packaging vector: pMDlg/pRRE	(Rubinson et al. 2003)
Lentiviral packaging vector: pRSV-Rev	(Rubinson et al. 2003)
Lentiviral vector: pFGEV16-Super-PGKHygro	(Vince et al. 2007)
Lentiviral vector: pF5xUAS-W-SV40-Puro	(Vince et al. 2007)
Lentiviral vector: pLKO.1-puro	(Geserick et al. 2009) (Sigma)
Lentiviral vector: pRRL-CMV-PGK-GFP-WPRE (Tween)	(Ricci-Vitiani et al. 2004)
Lentiviral vector: pLVTHM	Addgene, Plasmid #12247, D. Trono (von M. Wiznerowicz)
Lentiviral vector: pLVUT-tTR-KRAB	Addgene, Plasmid #11651, D. Trono
Lentiviral vector: pLVCT-tTR-KRAB	Addgene, Plasmid #11643, D. Trono
Lentiviral vector: pLVPT-rtTR-KRAB-2SM2	Addgene, Plasmid #11652, D. Trono
Lentiviral packaging vector: psPAX2	Addgene, Plasmid #16620, D. Trono

2.1.12. Prokaryotic cells

E.coli DH5α from Clontech (genotype: F-, φ80dlacZΞM15, Ξ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1)

E.coli M15 [pREP4] from Qiagen (M15 strain derived from E. coli K12 and has the phenotype NaIS, StrS, RifS, Thi-, Lac-, Ara+, Gal+, Mtl-, F-, RecA+, Uvr+, Lon+)

E.coli CH3-Blue from Bioline (genotype: F- ΔmcrA Δ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 ara Δ139 Δ(ara, leu)7697 galU galrpsL(StrR) nupG λ-)

E.coli TOP10F from Invitrogen (genotype: F´(lacIq Tn10 (TetR)) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG)

E.coli XL10 Gold from Stratagene (genotype: TetrD(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB lacIqZDM15 Tn10 (Tetr) Amy Camr])

2.1.13. Eukaryotic cells

HEK 293T: Human Embryonic Kidney 293 that stably express the large T-antigen of SV40) purchased from the American Type Culture Collection (ATCC)

HaCaT: Transformed Keratinocytes provided by P. Boukamp (DKFZ, Heidelberg, Germany, (Boukamp et al. 1988))

THP-1: Human leukaemia cell line (J. Tschopp)

DC2.4: Immortalisation of bone marrow cells under GM-CSF conditions, myc and raf oncogenes (Shen et al. 1997)

XS52: Mouse dendritic cell line

BO97.11: Mouse T-cell hybridoma that expresses the OVA-spezific T-cell-receptor (M. Lutz)

DO11.10: Mouse T-cell hybridoma that expresses the OVA-spezific T-cell-receptor and marginal CD4 (F. Haag)

FGK45: Rat B-cell hybridoma that produces agonistic CD40 (A. Eggert)

muGM: Mouse B-cell hybridoma that produces GM-CSF

7D4-E5: Rat B-cell hybridoma that produces anti mouse CD25

EL-4 IL4: Mouse B-cell hybridoma that produces mouse IL-4

Primary human keratinocytes were isolated from human foreskin of different patients (Donor 11 and 12). Primary murine ovalbumin specific T cells were isolated from spleen of C57BL/6-Tg(TcraTcrb)425Cbn/J animals with Pan T Cell Isolation Kit and primary bone marrow cells were isolated from tibia and femur of C57BL/6J animals and further differentiated into DCs.

2.1.14. Animals

C57BL/6J and C57BL/6-Tg(TcraTcrb)425Cbn/J (OT2) mice were purchased from Jackson Laboratories (California, USA).

B6.129-Cflar^{tm1Ywh}/J from Jackson Laboratories (California, USA); homozygous animals entitled as cFLIP-flox/flox mice and heterozygous animals entitled as cFLIP-flox/wt mice were backcrossed over 12 generations to C57BL/6J background (Zhang and He 2005).

2.1.15. Culture media for bacterial cells

LB-Agar (Lennox)	Roth
LB-Medium (Lennox)	Roth
SOC Medium	Sigma

All media were autoclaved, upon preparation, at 121°C for 15 minutes. Antibiotics were added after cooling down. The additives kanamycin (Kan) and ampicillin (Amp) dissolved in distilled water were filtered with a 0.2 µm filter-unit and stored at -20°C until the usage. The final concentrations were: 0.1 mg/ml for Amp and 0.05 mg/ml for Kan.

2.1.16. Cell culture media and reagents for cells

Dulbecco´s Modified Eagle Medium (D-MEM)	Invitrogen
RPMI 1670	Invitrogen
CnT-07	CellNTec

The media for eukaryotic cell culture were purchased from Invitrogen (DMEM, RPMI) supplemented with heat inactivated (56°C, 30 min) fetal bovine serum (Gibco) and the following additives if needed for cell culture.

Ultra Low IgG Fetal Bovine Serum	Invitrogen
HEPES	Sigma
Hygromycin B	MERCK
MEM Amino Acids	PAA
MEM Vitamins	PAA
Dulbecco´s Phosphate-Buffered Salines (D-PBS)	Invitrogen
Puromycin dihydrochloride	Sigma-Aldrich
Sodium Pyruvate	PAA
Trypsin EDTA	PAA
Zeocin™	Invitrogen
Penicillin / Streptomycin	PAA

2.1.17. Generally used buffers

1 x PBS	2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4, pH 7.4
1 x T-PBS	1x PBS, 0.1% Tween 20

2.1.18. Buffer and reagents for protein biochemistry

5 x Laemmli sample buffer	60 mM Tris-HCl (pH 6.8), 2% SDS, 10%l Glycerol, 5% β-mercaptoethanol, 0.01% Bromophenol blue
Antibody-buffer	3% nonfat dry milk, 1.5% BSA in 1 x T-PBS
Blocking buffer	5 % nonfat dry milk, 3% BSA in 1 x T-PBS
SDS Running Buffer (MES)	50 mM MES, 50 mM Tris-base, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.3
SDS Running Buffer (MOPS)	50 mM MOPS, 50 mM Tris-base, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.7
Transfer buffer	25 mM Tris, 192 mM glycine, 10% methanol

Ponceau S Solution	Sigma
Coomassie Brilliant Blue G-250	BioRad
Restore™ Western Blot Stripping Buffer	ThermoScientific

Cell lysis buffer for isolation of total cell proteins (30 mM TRIS-HCL (pH 7.5), 120 mM NaCl, 10% Glycerol, 1% Triton X, 2 tablets Complete (Protease Inhibitor) per 100 ml)

2.1.19. Buffer and reagents for molecular biology

DNA sample buffer	30 % (v/v) Glycerine, 50 mM EDTA, 0.25 % Bromophenol-blue, 0.25 % Xylene Cyanol
TAE buffer	40 mM Tris Base, 20 mM Acetic acid, 10 mM EDTA
Crystal violet staining solution	0.5 % crystal violet, 20 % methanol
Hypotonic fluorochrome solution	Sodium citrate 0,1% (w/v), Triton X 100 0.1 % (v/v), PI 50 µg/ml
Ethidium bromide solution	10 mg/ml in dH_2O
AnnexinVCy5 staining solution 10x	0.1M HEPES (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$

2.2. Methods

2.2.1. Molecular biological methods/Cloning

All molecular biological work was carried out corresponding to standard protocols delivered by the manufacturers. All methods are described in the literature in detail: Current Protocols in Molecular Biology (Ausubel et al. 1990) and Molecular Cloning (Sambrook et al. 1989). Therefore, only a brief description will be given and the modifications will be described in more detail if this applies.

2.2.2. Restriction of DNA

The restriction digestion reaction was performed in 20 µl mix, containing 10-15 U of respective enzyme, compatible buffer and around 1 µg of analysed DNA solved in water. The reaction was performed for 1 - 1.5 h on 37°C.

2.2.3. DNA agarose gel electrophoresis

DNA fragments were separated according to their size by one-dimensional agarose (0.8 - 1.2 %) gel electrophoresis. To visualize the DNA under UV light, 5 - 10 µl ethidium bromide solution was added before gel polymerization. The DNA samples were prepared in DNA sample buffer and were loaded onto the gel. Gels were run at 3 - 5 V/cm, depending on the required quality, in TAE buffer. The DNA fragments were visualized under UV-light and photographed.

2.2.4. DNA extraction and multiplex PCR

Genomic DNA from LV transduced BMDCs was extracted using an isolation kit (DNeasy Blood & Tissue Kit Qiagen, Hilden, Germany) as per manufacturer's instructions. Multiplex PCR (QM-PCR) was performed in a final volume of 20µl using

DreamTaq DNA polymerase (Thermo Scientific/Fermentas, Europe) in the FlexCycler (Analytikjena, Germany). The following primers were used: F1 5'-CAT GAG CAC TGA GGG ACA CAG CAC-3'; R1 5'- CGG AGT TTG CTA CAG GAA GGC CAC-3'; R2 5'- ACA CCA CCA GGG GCT GAC AAT AGA-3'.

The initial step of denaturation was 15 min at 95°C, followed by 35 cycles consisting of 30 s at 95°C, 45 s at 65°C, and 1 min at 68°C, followed by an extension period at 72°C for 10 min. Amplified products were analysed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide.

2.2.5. Generation of inducible protein overexpression constructs

DNA fragments of interest were purified subjected to enzymatic digestion and ligated with T4 DNA ligase to the pre-digested and dephosphorylated vector. The ligations were performed at 16-20°C for 2-8 h. The used DNA fragment/vector ratio was 3:1 (sticky end cloning). To select for positive clones, the ligated fragment-vectors were transformed into competent bacterial cells and plated on antibiotic containing agar plates for bacteria colony formation. The DNA of formed bacteria colonies was isolated for restriction digestion, sequencing (by (Agowa) LCC genomics) and maxi plasmid preparations.

2.2.6. Generation and stable siRNA expression (example cFLIP)

For generating of the siRNA constructs corresponding to the mouse cFLIP gene, an 29mer oligonucleotide containing cFLIP targeting sequences was annealed to a 68mer oligonucleotide oligomers (cFLIP, nucleotide start position + 395: (cgc gtc ccc AGA GCT TAG ATC AGA ACG ATG TAT CCT CC c caa GGA GGA TAC ATC GTT CTG ATC TAA GTC CT t ttt tgg aaa t). The annealed oligos were introduced into pLVTHM lentivector (Addgene/D.Trono). To produce drug-controllable RNA interference (RNAi) of cFLIP expression, the complete H1-RNA promoter and the siRNA cassette were subcloned at *MscI* and *XhoI* sites downstream from GFP open reading frame into tTRKRAB lentiviral vector (Addgene/D.Trono).

The resulting vectors or control empty vector were transfected into the 293T HEK cell line exactly as previously described (14). The concentrated lentivirus was used for primary DC transduction.

2.2.7. Heat shock transformation

The DNA ligation mixture was incubated with 100 µl of chemically competent bacteria cells for 30 min on ice. Heat shocking for 45 sec at 42°C was followed by incubation on ice for 5 min. Then 400 µl of SOC Medium was added and the tube incubated at 37°C for 1 h shaking speeding bacterial shaker (750 rpm). Bacterial cells were pelleted by centrifugation at 1000 g for 1 min and the supernatant was decanted. The pellet was resuspended by vortexing in the remaining drops of liquid. The entire suspension was plated on LB-Agar plates containing the respective antibiotics. Plates were incubated over night at 37°C.

2.2.8. Plasmid isolation

DNA plasmids were purified using Plasmid Kits and following manufactures protocols. The DNA concentration was determined by spectrophotometrical quantification at 260 nm by A_{260} * 50 = x µg/µl.

2.2.9. Cell biological methods /cell culture techniques

Cells were cultured in their respective media in 5% CO_2 at 37°C and a humidity of 95%. All media were pre-incubated at 37°C before addition to cells.

Cells	Medium
HEK 293T	D-MEM, 1 % HEPES, 1 % Sodium Pyruvate, 10 % heat inactivated FCS
HaCaT	D-MEM, 1 % HEPES, 1 % Sodium Pyruvate, 10 % heat inactivated FCS
DCs	RPMI, 10 % heat inactivated FCS, 1 % HEPES, 1 % Sodium Pyruvate, 1 % Pen/Strep, 50 µM β-Mercaptoethanol, 10 % muGM supernatant
TCs	RPMI, 10 % heat inactivated FCS, 1 % HEPES, 1 % Sodium Pyruvate, 1 % Pen/Strep, 50 µM β-Mercaptoethanol
Macrophages	D-MEM, 10% heat inactivated FCS, 1 % Pen/Strep, 30 µg/ml mCSF
DC2.4	RPMI, 5 % heat inactivated FCS
XS52	IMDM+10 % heat inactivated FCS, 1 % Pyruvat, 10% NS47-supernatant, 10 % muGM-supernatant
Primary keratinocytes	CnT-07 including the supplements
muGM, FGK45, THP-1	RPMI, 10 % heat inactivated FCS, 1 % Sodium Pyruvate
7D4-E5, EL4-IL4	RPMI, 10 % heat inactivated FCS, 1 % Sodium Pyruvate

2.2.10. Ca-phosphate mediated Transfection of vector-DNA

Cells mainly HEK 293T cells were grown in 10 cm plates in their medium. Then the medium was changed for 6 ml of respective medium per plate supplemented with 25 µM Chloroquine followed by 40'-1h incubation at 37°C in 5% CO_2. At the same time 32 µl of 2 M $CaCl_2$ was mixed with 20 µg DNA up to a final volume of 300 µl (per plate). This mix was added drop wise into 300 µl of 2x HBS buffer, followed by 30'-40' incubation on RT. Then the resolution mix with the Ca-DNA precipitate was added onto the plate and incubated over night at 37°C in 5 % CO_2 atmosphere before exchange of growth media.

2.2.11. Generation and purification of stimulating cytokines

For generation of CD95L-Fc (the construct was kindly provided by P. Schneider, (Bossen et al. 2006)) the corresponding construct, that encodes the hemagglutinin signal peptide, the Fc portion of human immunoglobulin G (IgG) (aa 108 to 338 of accession number AAC82527, excluding the stop codon), a linker sequence (RSPQPQPKPQPKPEPEGSLQ), and CD95L (aa 139 to 281), was transiently expressed in HEK 293T cells, cultured in medium containing IgG stripped FCS. Supernatants were harvested, filtered (0.22 µm, Millipore) and kept on 4°C for further usage (especially for the usage in special cell culture media for example muGM supernatant) or frozen down. The activity of purified protein was tested by functional assays (e.g. Cristal violet staining upon stimulation of the cells). One unit of Fc-CD95L was determined as a 1:500 dilution of the stock Fc-CD95L supernatant, and one unit/ml of Fc-CD95L supernatant was sufficient to kill 50 percent (LD50) of A375 melanoma cells, as recently described (Geserick et al. 2008). Caspase activity was completely blocked by addition of zVAD-fmk (Bachem, Weil am Rhein, Germany).

2.2.12. Production of lentiviral supernatants

Briefly, to generate lentiviral supernatants e.g. 2-2.5x10^6 of HEK293T cells per 10cm plate were transfected with 15 µg lentiviral packaging plasmids (3rd generation: pMDL g/p RRE - 10µg + pRSV-Rev - 5µg) together with the 20 µg transfer vector (e.g. pFGEV16-Super-PGKHygro, pF5xUAS-W-SV40-Puro, Tween or pLVUT-tTR-KRAB vectors containing molecules of interest or respective shRNA) by calcium phosphate precipitation. The supernatants containing viral particles were harvested 24 h post-transfection and collected over 2 days. Following filtration (45 µm filter, Schleicher&Schuell, Dassel, Germany) and concentration by centrifugation (19500 g, 2 h, 12°C) virus-pellet was dissolved in RPMI containing 5 % FCS, aliquoted and shock-frozen with liquid nitrogen and stored at -80°C until use.

2.2.13. Titration of lentivirus

For calculation of virus titer in GFP expressing supernatants 20000 THP-1 cells are plated in duplicates in 100 µl medium/well of 96 well plate. Cells were spin-infected with 7 different dilutions per viral supernatant in 1:3 or 1:2 dilution steps (starting for what?). 24 hours post transduction cells were washed twice and plated in 6 well plates for expansion. 18 to 24 hours later cells were analysed by FACS for GFP expression the percentage from linear values (usually between 5 and 10 %) was read. The titer is the number (percentage) of cells infected by a given volume and cell number after 48 hours in TU/ml (www.lentiweb.com)

2.2.14. Indirect method for titration of non GFP-expressing viral supernatants

To calculate the amount of viral particles in non-GFP expressing but selectable supernatants a crystal violet staining of HaCaT cells was performed. Briefly, cells were spin-infected with 8 different dilutions per viral supernatant in 96-well plates. 48 hours post transduction cells were selected with hygromycin and/or puromycin for 5 days. 18 to 24 hours after selection time plates were washed two times with water and stained by 50 µl of Crystal violet solution, added per well. Staining was performed for 20 min at room temperature followed by several washing steps with water. Plates were air dried and 200 µl methanol was added per well. After 30 min incubation at RT to solve the living cell-incorporated dye, the optical density of the wells was subsequently measured by a plate reader (Victor3; 1430 Multilabel counter, PerkinElmer). The optical density of control cultures was normalized to 100 % and compared with stimulated cells. The titer is here the number (percentage) of surviving infected cells by a given volume and cell number after 7 days in TU/ml.

2.2.15. Lentiviral infection of cells

The virus containing supernatants were added to target cells in the presence of 5 µg/ml hexadimethrine bromide (polybrene). Mainly all cells e.g. DC2.4 cells were spin-infected (2300 rpm, 30°C, 1.5 h). 18 - 24 h later, cells were washed with PBS

and viral particle containing supernatants were replaced by fresh medium. Cells were either selected with hygromycin (100-150 µg/ml for 1 week) and/or puromycin (1 µg/ml for 3 days), or were incubated with doxycyclin for 5 days. Cells were subsequently tested for expression/downregulation of the respective molecules. In case of the tamoxifen-inducible system testing was performed 24 h after induction with 10 to 100 nM 4-HT (4-Hydroxytamoxifen).

2.2.16. Lentiviral infection of BMDCs

For lentiviral transduction DCs were plated in RPMI medium supplemented with mGM-CSF and polybrene (5-8 µg/ml). Concentrated LVs at a multiplicity of infection (MOI) of 50-80 were added to the plated cells at two days following transduction. The cells were incubated for 3-4 hrs at 37°C with viral supernatants followed by addition of 10 ml media for overnight incubation. After each transduction step virus containing supernatant was discarded and fresh medium was added. At 3 days of post-transduction, siRNA expression was induced by doxycyclin and the cells were further cultivated for another 5 days. If gene expression e.g. dnASC was not inducible, cells were analysed 48 to 72 hours of post-transduction and further experiments were performed.

2.2.17. Cell lysate preparation for Western blot analysis

For generation of total cell lysates parental and virally transduced cells were washed twice with ice-cold 1 x PBS and lysed for 30 min on ice by the addition of lysis buffer. Cellular debris was removed by centrifugation at 20,000 g for 20 min. The total amounts of isolated cell proteins were quantified by Bradford assay. 3-10 µg of total cellular proteins were supplemented with 5 x Laemmli sample buffer and boiled at 95°C for 10 min.

To test if no effects on caspase expression and activation were visible due to our experimental system and lysis conditions, we performed cell lysis with zVAD-fmk within the lysis buffer twice. We could not detect any changes or differences in Western Blot analysis (data not shown) and so did not change to cell lysis under zVAD-fmk conditions.

2.2.18. Western Blot analysis

Total cellular proteins were separated by SDS-PAGE on 4-12% gradient gels (Invitrogen, Karlsruhe, Germany) using SDS Running Buffer and Invitrogen Power Ease 500 system followed by a transfer to a PVDF membrane using Transfer buffer. Membranes were incubated for 1 h in blocking buffer, washed with 1 x T-PBS and incubated with primary antibodies at 4°C overnight. After washing with 1 x T-PBS, blots were incubated with HRP-conjugated isotype-specific secondary antibody in 2.5 % milk in 1 x T-PBS for 1 hour at room temperature. Afterwards membranes were washed with 1 x T-PBS, bands were visualized with ECL detection kits (Amersham, Freiburg, Germany) by chemiluminescence and films (AGFA Curix HT and Amersham Hyperfilms) or with INTAS machine. For further analysis of more proteins, membranes were washed twice with T-PBS and stripped with Restore™ Western Blot Stripping Buffer according to manufactures protocol for 5 – 15 minutes at room temperature. After stripping membranes were washed 5 x with T-PBS and blocked with blocking buffer for 1 h. Incubation with next primary antibody was performed after washing with T-PBS for 3 times. This procedure was repeated up to 4 times per membrane.

2.2.19. Visualization of proteins: Coomassie, Silver and Ponceau staining

To visualize proteins in a gel Coomassie- or silver staining was performed according to standard protocols. The gradient gels were incubated for 30 min in Coomassie stain and destained by washing in 10 % acetic acid solution. The more sensitive silver staining was performed if needed. Therefore the protein gels were fixed in 30 % ethanol, 10 % acetic acid for 1 h or overnight at RT. Fixed gels were washed 3 times for 20 min with 50 % ethanol and sensitized with 0.02 % sodiumthiosulfate-pentahydrate for 2 min. After 2 time washing for 2 min, proteins were stained with 0,1 % cold silver nitrate solution for 30 min. Protein bands were visualized after 2 more washes (1 min, dH_2O) with developer solution (0.04 % formaldehyde, 2 % sodium carbonate) until desired intensity of bands was achieved. Staining was stopped with 1 % acetic acid; gel was washed with 50 % methanol and scanned.

To visualize proteins on a PVDF membrane a Ponceau staining was performed. Briefly, membrane was incubated for 20 min in Ponceau S solution (Sigma); protein bands were visualized by washing carefully with T-PBS and scanned if desired. After destaining the membranes with 0.1 M NaOH for 1 min Western blotting can be performed as previously described.

2.2.20. Hypodiploidy analysis

Subdiploid DNA content was analysed as previously described (Wachter et al. 2004). Briefly, cells were stimulated with the indicated reagents for indicated timepoints. After pelleting (200-400 g 5 min) and 2 washing steps with cold PBS, cells were resuspended in 500 µl of cold hypotonic fluorochrome solution (Sodium citrate 0,1 % (w/v), Triton X 100 0,1 % (v/v), PI 50 µg/ml) and kept in the dark at 4°C. Diploid and subdiploid content was measured by FACScan analysis 36 - 48 hours later.

2.2.21. Immunofluorescence microscopy

For the detection of the GFP-reporter gene expression, cells were analysed with fluorescence microscopy using a Zeiss Axiovert HBO 50 microscope (Jena, Germany). Digital images were processed in an identical manner using the advanced SPOTSOFTWARE, Version 4.6 (Diagnostic Instruments Inc, MI, USA). For detection of nuclear morphology and integrity of the cell membrane, $5x10^4$ cells seeded in 12-well plates were incubated with Hoechst 33342 (5µg/ml; Polysciences Europe, Eppelheim, Germany) and SYTOX®Green (5pM; InvitrogenTM, Molecular ProbesTM, Eugene, Oregon, USA) for 15 min at 37°C immediately followed by phase contrast or fluorescence microscopy. Digital images were processed in an identical manner using the advanced SPOTSOFTWARE, Version 4.6 (Diagnostic Instruments Inc, MI, USA).

2.2.22. Ligand affinity precipitation of Receptor complexes (DISC)

For the precipitation of the CD95L DISC, 5 x 10^6 cells were used for each condition. Cells were washed once with medium at 37°C and subsequently preincubated (if needed) for 1 h with 5-10 µM zVAD-fmk (Bachem, Weil am Rhein, Germany) at 37°C. Subsequently cells were treated with 250 U/ml CD95L-Fc for 30 min or, for the unstimulated control, in the absence of ligands. Receptor complex formation was stopped by washing the cells four times with ice-cold PBS. Cells were lysed on ice by addition of 1 ml lysis buffer. After 1 hour lysis on ice, the lysates were centrifuged two times at 20,000 x g for 5 min and 30 min, respectively, to remove cellular debris. A minor fraction of these clear lysates were used to control for the input of the respective proteins.

For the precipitation of the CD95 receptor and stimulation-dependent recruited proteins, CD95L-Fc was added to the lysates prepared from non-stimulated as well as stimulated cells to precipitate the receptor-interacting proteins. The levels of receptor precipitates were compared in all experiments by Western blotting for CD95.

Receptor complexes were precipitated from the lysates using 40 µl protein G beads (Roche, Mannheim, Germany) for 16 - 24 hrs on an end-over-end shaker at 4°C. Ligand affinity precipitates were washed 4 times with lysis buffer before the protein complexes were eluted from beads, dried by the syringe needle, by addition of standard reducing sample buffer and boiling at 95°C. Subsequently, proteins were separated by SDS-PAGE on 4-12% NuPAGE gradient gels (Invitrogen, Karlsruhe, Germany) before detection of DISC components by Western blot analysis.

2.2.23. Generation of primary murine dendritic cells

Large quantities of highly pure dendritic cells (DCs) were generated by cultivation of bone marrow cells in the presence of GM-CSF as previously described (Lutz et al. 1999). Briefly, tibiae and femurs of sacrificed C57/BL6 mice were isolated and bone marrow cells were washed out with PBS using a syringe. Single cell suspensions were obtained by gently pipetting cells up and down and filtering through a cell strainer (100 µm, Nylon, BD). Isolated cells were either directly frozen in culture

media containing 20 % of FCS and 10 % DMSO or cultured in the presence of recombinant murine GM-CSF in a density of 1 x 10^5 cells / ml in culture Petri dishes (BD) after lysis of erythrocytes. To get rid of macrophages etc. on the next day only suspension cells were plated into new plates in fresh media. Exchange of media was made at day 6 and 8. After 6 days bone-marrow-derived monocytes have differentiated into dendritic cells, surface expression of DC-markers was analysed by FACS analysis.

After generation of immature murine dendritic cells e.g. 1 µg/ml LPS was used as substance mimicking bacterial induced maturation or a cocktail containing 10 % FGK45 supernatant, 20 ng/ml murine TNF-α, 1 % EL-4-IL-4 supernatant and 1 µg/ml PGE-2 mimicking viral induced maturation.

2.2.24. Generation of primary murine T cells (TCs)

Single cell suspension from spleen was generated from OT2 mice (purchased by Jackson Laboratory). Briefly, spleen cells were isolated and smashed over a 100 µm nylon cell strainer (BD), filtered twice and washed with cold PBS. Cells were stained with CFSE for 10 min at 37°C and staining was stopped with MACS Buffer (PBS, 2 mM EDTA, 0.5 % BSA). Cells were washed and loaded with antibodies against non-TCs (CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, Ter-119) and paramagnetic beads for magnetic cell separation according to Pan T Cell Isolation Kit II (Miltenyi) protocol. Untouched mouse T cells were isolated as negative fraction from LS Columns (Miltenyi) suitable for the depletion of strongly magnetically labeled cells. TCs were washed twice after separation and cultured in their medium upon use.

2.2.25. *in vitro* TC proliferation assay

In brief, naïve CD4+ T cells were prepared from splenocytes of transgenic OT2 mice. In the allogenic mixed lymphocyte reaction assay, LPS-treated or untreated, OVA protein (grade III; Sigma) or OVA peptide pulsed DCs were co-cultured at ratios 1 (DC): 5 (TC) and 1 (DC: 10 (TC) in 96-well plates in a final volume of 200 µl TC-

medium / well. After 60 hours T cell proliferation was measured (CFSE content) using the FACS Canto II System (BD), aliquots of supernatants were collected and frozen down for further analysis of cytokine content.

2.2.26. Endocytosis analysis

FITC-Dextran was purchased from Molecular Probes, Inc. Cells were grown in culture medium in the presence or absence of maturation stimuli. 24 hours later, cells were incubated at 37°C or at 0°C for 1 h with 200 µg/ml FITC-Dextran (FITC-DX). Uptake was stopped by adding ice-cold FACS-buffer followed by three washing steps with ice-cold PBS containing 5 % BSA. Cells were then analysed by flow cytometry using a BD FACS Canto (Becton-Dickinson, San Jose, CA); the uptake was quantified as mean fluorescence intensity (MFI).

2.2.27. Determination of cytokine secretion

For measurement of cytokines in cell supernatants, the Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex kit (eBiosciences) was used according to the manufacturer's instructions using a FACS Canto II System (Becton Dickinson). IL-2, IL-1β, IL-18 and IL-33 levels were determined by enzyme-linked immunosorbent assay (ELISA) (eBiosciences, R&D Systems, Biozol).

2.2.28. Flow cytometry

For phenotypic analysis of cell populations, staining was performed according to standard immunofluorescence techniques. Briefly, aliquots of cells (100000-250000 cells/sample) were incubated for 1 h on ice with indicated primary antibodies, washed ones with PBS and were then exposed for 45 minutes to PE-conjugated secondary antibodies. After 2 more washing steps with PBS, the cells were analysed by flow cytometry (FACS Canto II, BD). Isotype matched monoclonal antibodies were used as controls, propidium iodide or 7-AAD were used as marker for dead cells. Data were analysed with FACS Flow Software.

For sorting of defined cell populations (e.g. GFP positivity), cells were collected, washed with PBS, counted, filtered with a 50 µm CellTrics® filter (PARTEC), resuspended in sorting buffer (PBS, 2% BSA, 2 mM HEPES, Pen/Strep) and carried over to the FACS Sorting Core Facility Mannheim. Desired cell populations were collected by FACSAria (BD), dead cells were gated out using SYTOX®red (Invitrogen) dye staining during sort. Sorted cells were washed twice with their medium after sorting and cultured until use.

2.2.29. RNA isolation and realtime Q-PCR

Total RNA extraction was performed using RNeasy Kit (Qiagen). cDNA was synthesized in 20 µl using a mixture of oligo dT primers and random nonamers in a ratio of 1:10 and SuperScript II Reverse Transcriptase (Invitrogen).

Primers were designed using Primer3 software (available at http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi). RT qPCR analyses for the genes encoding interleukin-1β (IL-1β), interleukin.18 (IL-18, interleukin-33 (IL-33), murine cFLIP, caspase-8 and β-actin were performed in a final volume of 25 µl using KAPA SYBR Fast qPCR (Peqlab) in an Mx3005P (Stratagene) real time termal cycler. The primers in following table x were used:

target	**sequence (5' – 3')**
ILbeta1-mfor1	acg ttc cca tta gac aac tg
ILbeta1-mrev1+2	aca ggt att ttg tcg ttg ct
ILbeta1-mfor2	tgg gat gat gat gat aac ct
ILbeta18-mfor1	agt aag agg act ggc tgt ga
ILbeta18-mrev1	aac tcc atc ttg ttg tgt cc
ILbeta18-mfor2	ctg aag aaa atg gag acc tg
ILbeta18-mrev2	tca cag cca gtc ctc tta ct
IL33-mfor1	ctg ttg aca cat tga gca tc
IL33-mrev1	cta gta gca cct ggt ctt gc
IL33-mfor2	cac taa aat cgg gta cca ag

IL33-mrev2	gat gct caa tgt gtc aac ag
β-actin for1	aga aaa tct ggc acc aca cc
β-actin re**v**1	ggg gtg ttg aag gtc tca aa
cFLIP for1	aga aaa atg cct gaa gaa ca
cFLIP rev1	cac ggt att cca caa atc tt
Caspase-8 for	ctg tga atg gaa cct ggt at
Caspase-8 rev	ctt ctt ccg tag tgt gaa gg
β-actin for2	gag caa gag agg tat cct ga
β-actin rev2	agg tct caa aca tga tct gg

Genes of interest and reference gene products were amplified individually under equal cycling conditions. HotStarTaq DNA Polymerase was launched by an initial 15 min at 95°C followed by 42 cycles of one step (denaturation) at 94°C for 15 sec, one step (annealing) at 55°C for 30 sec, and one step (extension) at 72°C for 30 sec. The specific amplification of a single product of the expected size was confirmed by melting curve analysis. Consecutive dilutions of cDNA (1, 1:5, and 1:25) were amplified for the construction of a standard curve (plotted as a logarithmic function of the cDNA dilution factor) and used for the calculation of the RT PCR efficiency.

The relative quantification for respective genes was calculated after dividing the standard curve value of one gene by that of the reference gene β-actin for each individual sample. Two reference genes were used for data normalization to account for possible variations as a result of maturation stimuli treatment. The effects on IL-1β, IL-18, IL-33, cFLIP and caspase-8 expression were calculated by analysing mean values obtained from three independent experiments. In two independent experiments, RNA was three times reverse transcribed and the cDNA from the three independent reverse transcriptions were assayed for RT qPCR in triplicates. The mean values obtained by the above explained procedure were compared for all different experimental conditions.

3. Results

3.1. Generation and characterisation of murine bone marrow-derived dendritic cells (BMDCs).

Dendritic cells (DCs) belong like monocytes, macrophages and B-lymphocytes to the antigen presenting cells (APC). In order to investigate *inter alia* the role of the inflammasome in DCs, a murine DC model system was established. To this end, BMDCs were isolated as described in Materials and Methods and their maturation and differentiation state as well as the contamination with other cell populations was tested.

Surface markers that allow determining the maturation status of the isolated BMDCs were analysed by flow cytometry before and after stimulation. CD11c served as specific dendritic cell marker and CD68 staining was the control for macrophage contamination. The maturation of the cells with either LPS (L) or with a cocktail (C) containing CD40L, PGE-2, IL-4 and TNF-alpha led to an upregulation of CD40, CD80, CD86 and MHC-II (Figure 7 A) and to a reduction of the endocytotic ability determined by the uptake of FITC-Dextran in comparison to unstimulated, immature DCs (I) (Figure 7 B). The positive staining of all three populations with CD11c confirmed DC differentiation. The negative staining for CD68 confirmed the lack of contamination with macrophages in the cell culture. Results indicated that isolated BMDCs are matured upon treatment with the used maturation agents, differentiated and pure and were thus suitable for the experiments in this work.

3.1.1. Characterisation of inflammatory signalling pathways in murine dendritic cells

In order to investigate the role of the inflammasome for maturation and antigen presentation, and for characterisation of the molecules involved in inflammatory

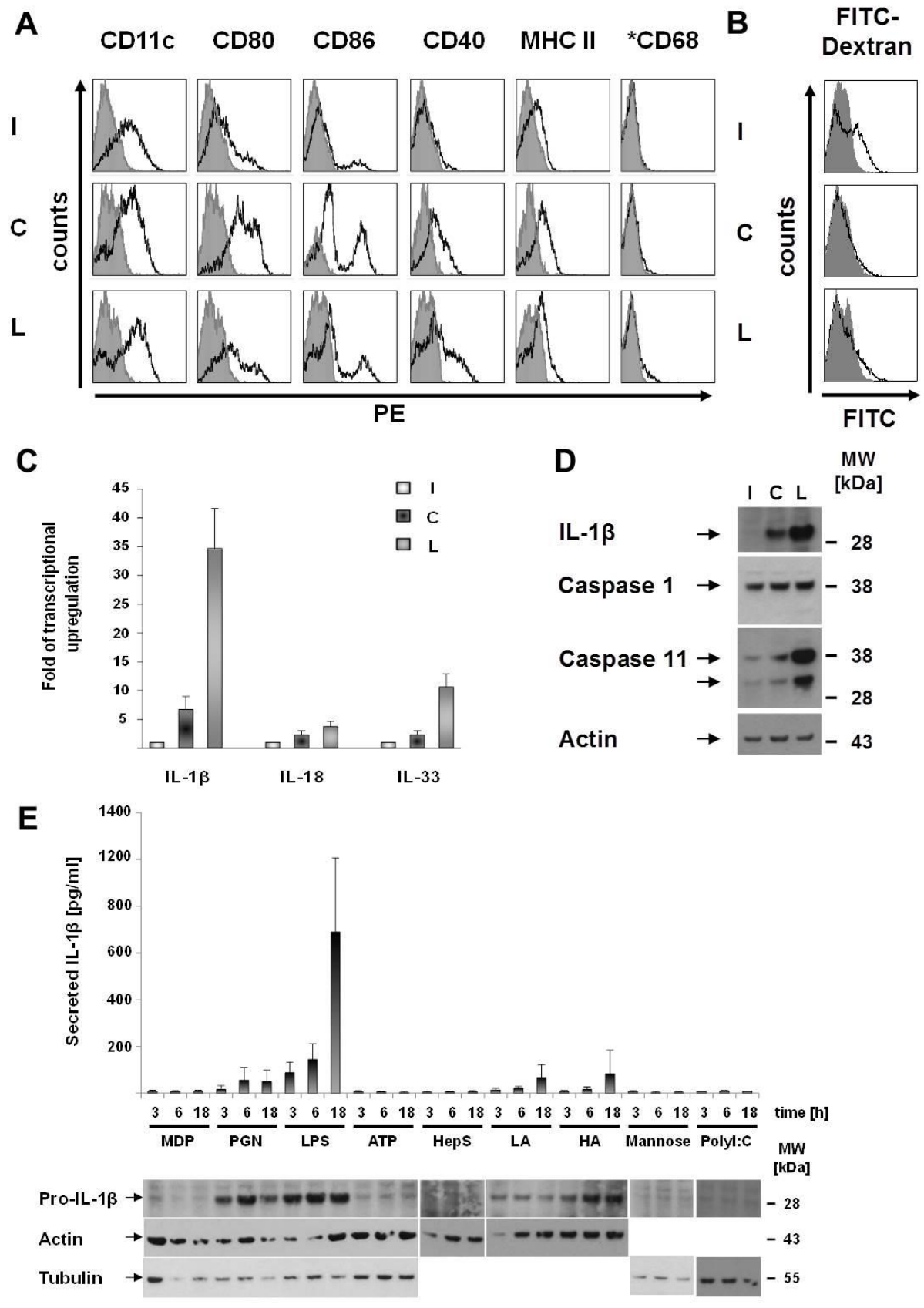
signalling pathways in BMDCs, I first analysed the expression of inflammatory components in mature and immature DCs.

The mRNA levels of the inflammatory cytokines IL-1β, IL-18 and IL-33 in respect to the maturation status of BMDCs were analysed. As shown in Figure 7 C the mRNA level of *IL1B*, *IL18* and *IL33* was increased in mature BMDCs, which was confirmed for IL-1β on the protein level (Figure 7 D), as well. Antibodies for IL-33 and IL-18 detection in Western Blot analysis were not yet available at the time. Furthermore, some of the inflammasome protein components like Caspase-1 and the Caspase-11 fragments p43 and p35 were detected in Western blot analysis in BMDCs with a high increase of both caspase-11 fragments in LPS matured population “L” (Figure 7 D).

So indeed, maturation of BMDCs leads to an upregulation of the inflammatory cytokines IL-1β, IL-18 and IL-33 and the inflammatory caspase 11, which indicates an activation of inflammasome-mediated signalling pathways.

Based on the knowledge that LPS as well as Heparan Sulfate (HS) and muramyl dipeptide (MDP) are recognized by TLR4, agents activating the TLR2, TLR3 and mannose receptor signalling pathway were tested for activation of inflammasome-mediated signalling pathways in DCs. Upon stimulation of BMDCs with different agents, an upregulation of the pro-IL-1β protein within the cell and a secretion of IL-1β were detected by ELISA only upon LPS, peptidoglycan (PGN), hyaluronic acid (HA) or lipoteichonic acid (LA) treatment in a time-dependent manner (Figure 7 E). So, this cytokine release after TLR4 and TLR2 stimulation of BMDCs demonstrates a clear activation of inflammasome-mediated signalling pathways in DCs upon incubation with some PAMPs.

Taken together, these results demonstrate that generated BMDCs are immature and can be matured by different stimuli, which leads *inter alia* to an upregulation of certain inflammatory molecules within the cells and to a release of the inflammatory cytokine IL-1β. So the dendritic cell system can be used to investigate the molecular mechanisms of inflammasome activation and signalling.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CD11c
CD80
CD86
CD40
MHC II
*CD68
I
C
L
counts
PE
B
FITC-Dextran
I
C
L
counts
FITC
C
Fold of transcriptional upregulation
45
40
35
30
25
20
15
10
5
I
C
L
IL-1β
IL-18
IL-33
D
MW [kDa]
I C L
IL-1β
Caspase 1
Caspase 11
Actin
28
38
38
28
43
E
Secreted IL-1β [pg/ml]
1400
1200
800
600
400
200
3 6 18 3 6 18 3 6 18 3 6 18 3 6 18 3 6 18 3 6 18 3 6 18 3 6 18
time [h]
MDP
PGN
LPS
ATP
HepS
LA
HA
Mannose
Polyl:C
MW [kDa]
Pro-IL-1β
Actin
Tubulin
28
43
55

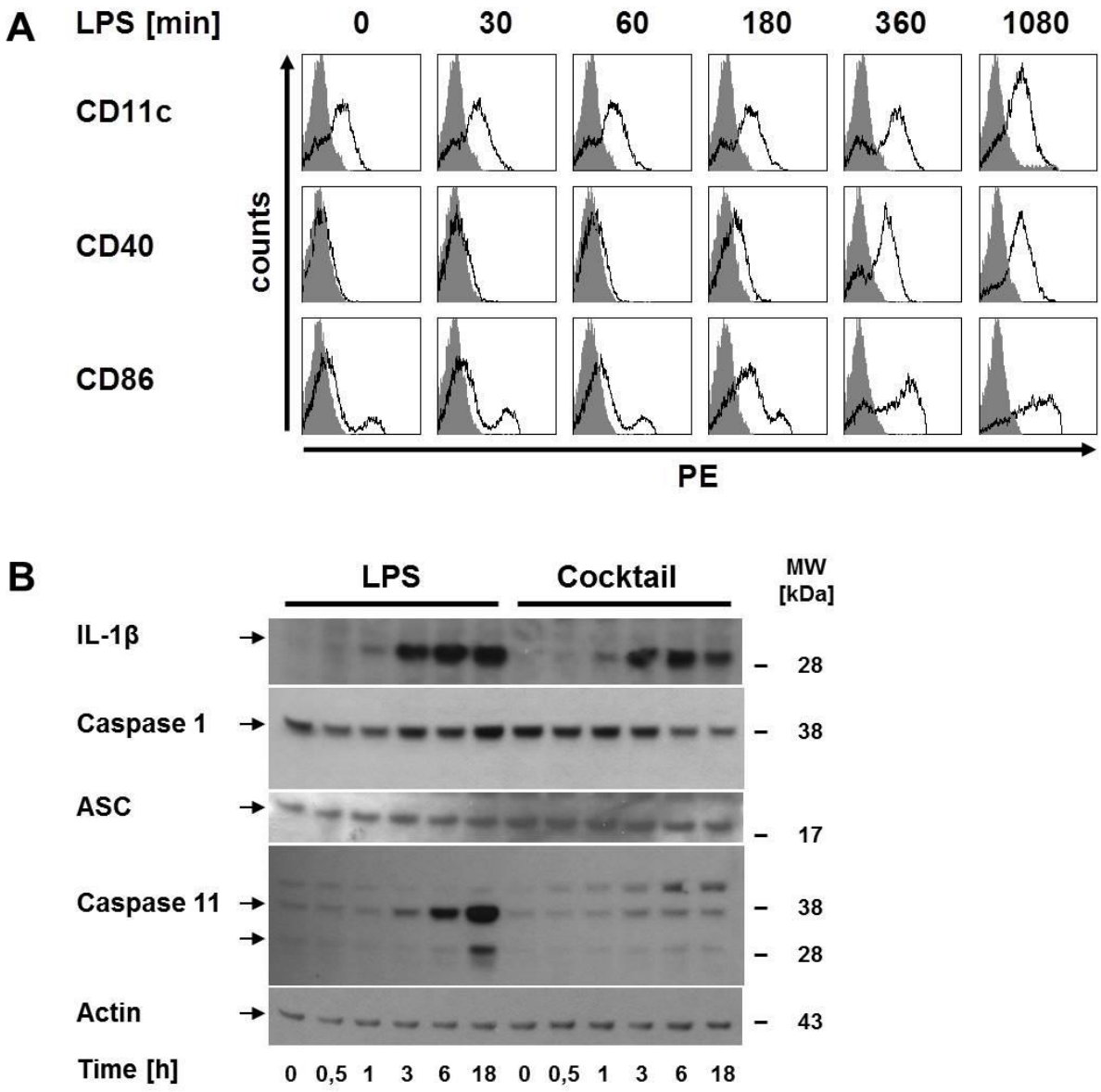
Figure 7

Figure 7: Characterisation of primary murine DCs upon different maturation stimuli.
Primary dendritic cells (BMDCs) were isolated from bone marrow cells of C57BL/6 mice and generated according to the method described by Lutz et al. in 1999 (Lutz et al. 1999). After 6 days of culture with GM-CSF immature (I) BMDCs were matured with 1 µg/ml LPS (L), 3 µg/ml MDP, 1 µg/ml PGN, 1 mM ATP, 1 µg/ml HepS, 1 µg/ml LA, 2 µg/ml HA, 2 mM Mannose, 1 µg/ml Poly I:C or a maturation cocktail (C) containing 1 % TNF-α supernatant, 1 % IL-4 supernatant, 10 % CD40L and 1 µg/ml PGE-2 overnight (16-18 hours) (Aiba 1998; Lee et al. 2002; Lipscomb and Masten 2002; Reis e Sousa 2006; Schmidt et al. 2012; Winzler et al. 1997). (A) DC and maturation marker surface expression was assayed by flow cytometry (black). Corresponding isotype controls of antibodies are shown in grey in the histograms. (B) Endocytotic ability was assayed by feeding with 1 mg/ml FITC-labeled Dextran and measured by flow cytometry. (C) The expression of inflammatory cytokines *IL1B*, *IL18* and *IL33* was assessed by quantitative real-time PCR. Transcript levels were normalized to *ACTB*. Expression levels are shown as fold increase compared with those of non-matured DCs. (D) Protein expression of pro-IL-1β, caspase-1 and caspase-11 was analysed by Western blotting, and expression of β-actin served as an internal control for equal loading of protein lysates. (E upper part) IL-1β secretion was measured by ELISA after different stimuli to induce BMDC maturation. Data are representative of three independent experiments and are expressed as mean values ± SE (E lower part) Protein expression of pro-IL-1β was analysed by Western blotting, and expression of β-actin or tubulin served as an internal control for equal loading of protein lysates.

3.1.2. The expression and secretion level of IL-1β correlates with the DC maturation status.

To test if the expression and secretion level of IL-1β directly correlates with the DC maturation, primary immature BMDCs were stimulated with 1 µg/ml LPS or the maturation cocktail (described in 2.2.23) for 30, 60, 180, 360 and for 1080 minutes. Surface marker analysis by flow cytometry confirmed DC maturation over time, namely the increase of CD11c, CD40 and CD86 surface expression (Figure 8 A). The expression level of IL-1β was determined by Western blotting and its secretion was measured by ELISA. Stimulation by LPS and cocktail resulted in an increase of IL-1β protein level within the cell after 1 - 3 hours (Figure 8 B) and to an advanced secretion of the cytokine starting at 3 hours after stimulation (Figure 8 C). The level of IL-1β itself differed between cocktail- and LPS-stimulation. Additionally, the expression of other inflammatory molecules upon maturation was investigated.

Interestingly, Caspase-11 expression was also enhanced after LPS stimulation after 3 to 18 hours whereas the expression of the adaptor molecule ASC and Caspase-1 were not affected by DC maturation (Figure 8 B). Thus, maturation of DCs along with activation of inflammasome-mediated signalling pathways is time-dependent and affects inflammatory molecules differently according to the maturation stimuli. Some of the inflammatory components like the adaptor molecule ASC are even not affected by the tested maturation methods. So, ASC is constitutively expressed in DCs, whereas the expression of e.g. the pro-inflammatory cytokine IL-1β gets upregulated upon maturation of DCs.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LPS [min]
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30
60
180
360
1080
CD11c
CD40
CD86
counts
PE
B
LPS
Cocktail
MW [kDa]
IL-1β
Caspase 1
ASC
Caspase 11
Actin
28
38
17
38
28
43
Time [h]
0 0,5 1 3 6 18 0 0,5 1 3 6 18
C
Cocktail
LPS
Secreted IL-1β [p/ml]
10000
1000
100
10
0
0,5
1
3
6
18
Maturation [h]

Figure 8

Figure 8: BMDC-maturation induced IL-1β secretion in time.
BMDCs were incubated with either 1 µg/ml LPS or the maturation cocktail for different time periods (0, 30, 60, 180, 360 and 1080 minutes). (A) DC marker (CD11c) and maturation marker (CD40 and CD86) surface expression was assayed by flow cytometry after LPS maturation. (B) Protein expression of the inflammatory molecules pro-IL-1β, Caspase-1, ASC and Caspase-11 was analysed by Western blotting, and β-actin served as an internal loading control. (C) The IL-1β secretion over time was measured by ELISA. The mean ± SE of three independent experiments is represented.

3.1.3. Stimulation of the P2X7 receptor increase IL-1β and IL-18 secretion.

Next, I tested if the maturation-induced cytokine secretion by DCs can be enhanced by stimulation of the $P2X_7$ receptor with adenosine triphosphate (ATP) or with uric acid (3.1.3.) like shown for monocytes and macrophages. Monocytes release endogenous ATP after LPS stimulation, which leads to the induction of IL-1β and IL-18 secretion in an autocrine way due to the activation of purinergic $P2X_7$ receptors by extracellular ATP followed by a K+ efflux (Franchi et al. 2007; Pelegrin et al. 2008).

Indeed, the experiments showed that upon maturation the IL-1β secretion was further increased after stimulation with ATP in BMDCs (Figure 9 A). The secretion of the inflammatory cytokines IL-1β and IL-18 after ATP stimulation occurred rapidly and reached a plateau phase upon 30-60 minutes stimulation (Figure 9 B). The amount of the cytokines differed between the three tested animals and was much higher for IL-1β as was for IL-18. In contrast, no IL-33 secretion was detected (data not shown).

To investigate if in our DC model the ATP-induced IL-1β secretion is mainly triggered by $P2X_7$ receptors, specific ATP derivatives were used. Adenosine 5´-triphosphate, periodate oxidized sodium salt (ox-ATP) was described to inhibit the $P2X_7$ receptor signalling while 2´(3´)-O-(4-Benzoylbenzoyl)adenosine 5´-triphosphate triethylammonium salt (bz-ATP) functions as specific activator. The bz-ATP derivate activated the $P2X_7$ receptor in a comparative way like ATP shown indirect by IL-1β secretion, while combination of both enhanced the cytokine release further. Ox-ATP partially inhibited the IL-1β secretion and was not showing any inhibitory effect upon co-stimulation with bz-ATP, possibly due to its weak affinity for the $P2X_7$ receptor and its slow kinetics. So, the detected induction or partial inhibition of the IL-1β release shows that the $P2X_7$ receptor is one key player for the cytokine release in DCs.

Interestingly, no differences were detected on protein level of Caspase-1, Caspase-11, ASC and pro-IL-1β upon maturation and stimulation with the ATP derivatives (Figure 9 D). Thus, DCs probably endogenously express inflammatory proteins upon maturation and a stimulation time of only 30 minutes with ATP or derivatives was not sufficient to detect effects neither on the internal IL-1β reservoir nor on other tested inflammatory proteins in Western Blot analysis.

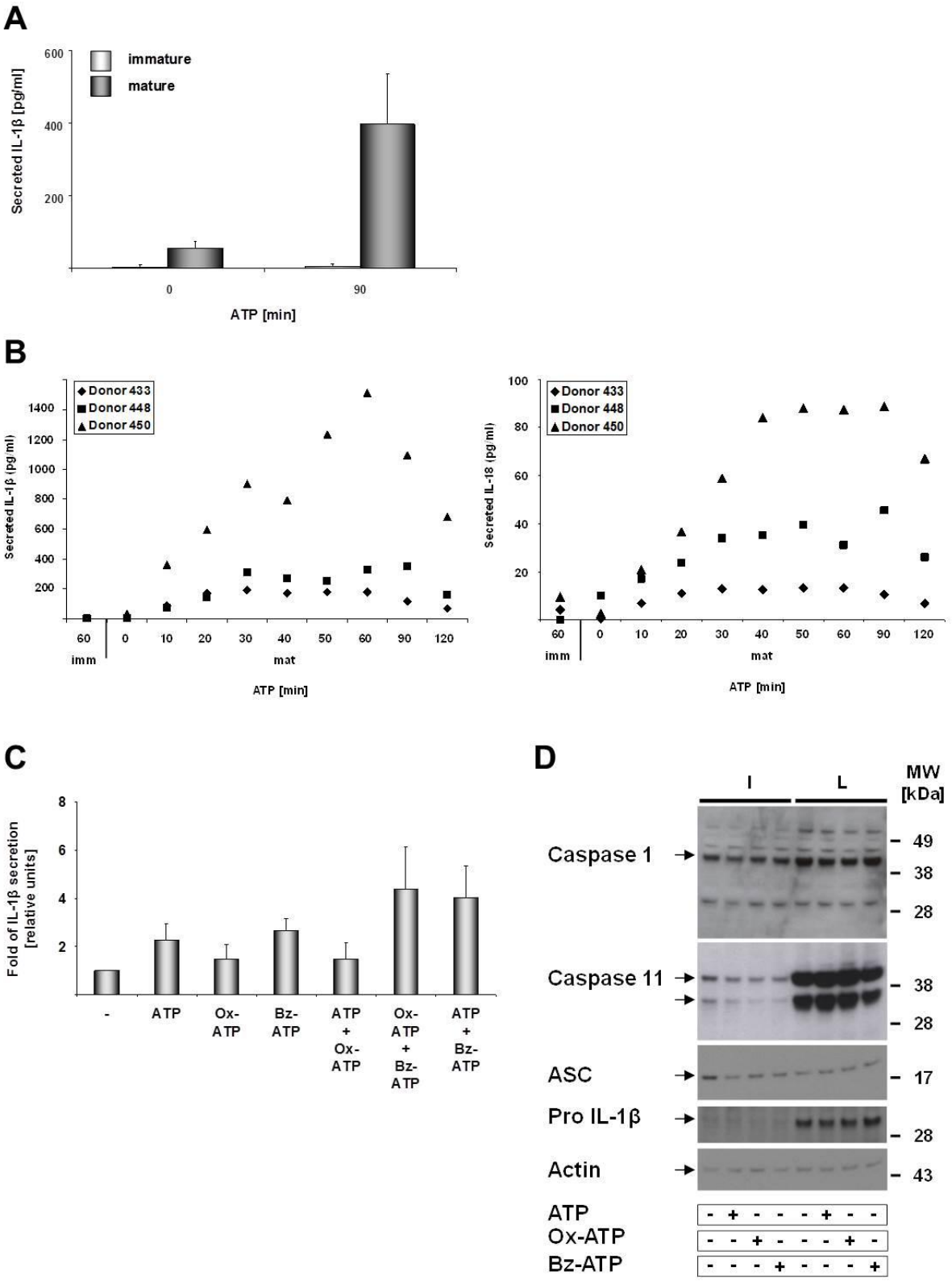

A
immature
mature
Secreted IL-1β [pg/ml]
600
400
200
0
90
ATP [min]
B
Donor 433
Donor 448
Donor 450
Secreted IL-1β (pg/ml)
1400
1200
1000
800
600
400
200
60
0
10
20
30
40
50
60
90
120
imm
mat
ATP [min]
Secreted IL-18 (pg/ml)
100
80
60
40
20
C
Fold of IL-1β secretion [relative units]
8
6
4
2
-
ATP
Ox-ATP
Bz-ATP
ATP + Ox-ATP
Ox-ATP + Bz-ATP
ATP + Bz-ATP
D
I
L
MW [kDa]
Caspase 1
49
38
28
Caspase 11
38
28
ASC
17
Pro IL-1β
28
Actin
43
ATP
Ox-ATP
Bz-ATP

Figure 9

Figure 9: $P2X_7$ receptor stimulation by ATP enhanced the secretion of IL-1β and IL-18.
BMDCs were matured with 1 µg/ml LPS and further stimulated with ATP or different ATP-derivatives for different time periods as indicated; followed by determination of cytokine release by ELISA (A, B, C). Immature and mature DCs were stimulated with 1 mM ATP for (A) 0 and 90 minutes and for (B) 0, 10, 20, 30, 40, 50, 60, 90 and 120 minutes. (C) Mature DCs were stimulated with 300 µM ATP, 300 µM ox-ATP, 300 µM bz-ATP, or a combination of those. (D) Protein expression of the inflammatory molecules pro-IL-1β, Caspase-1, ASC and Caspase-11 was analysed by Western blotting, and expression level of β-actin served as an internal loading control. Data are representative of three independent experiments and are expressed as mean values ± SE.

3.1.4. Monosodium urate does not enhance the IL-1beta secretion like ATP in DCs.

I speculated that like ATP, which was shown to induce IL-1β in macrophages, monosodium urate crystal (MSU) stimulation leads to IL-1β secretion of LPS primed (mature) DCs. To test this hypothesis, I generated macrophages (BMDMs) in parallel to DCs (BMDCs) from the same pool of bone marrow cells. Surface marker were analysed by flow cytometry; CD11c served as DC marker and CD68 as macrophage marker (Figure 10 A). The macrophages were also positive for CD80, CD86, CD40 and MHCⅡ. Both cell types expressed the inflammatory molecules of interest, Caspase-1, Caspase-11, ASC and IL-1β (Figure 10 B). BMDCs and BMDMs showed an upregulation of Caspase-11 upon LPS maturation. The total protein levels for ASC and IL-1β were lower in BMDMs than in BMDCs. No significant differences could be seen for Caspase-1, ASC and pro-IL-1β upon ATP or MSU stimulation. In mature BMDCs as well as in mature BMDMs the protein level of the p30 of Caspase-11 was enhanced by ATP stimulation and stronger by MSU (Figure 10 B).

Interestingly, only BMDCs were able to enhance their IL-1β secretion upon ATP stimulation. The IL-1β secretion of LPS primed macrophages was not increased upon further stimulation with ATP or MSU (Figure 10 C) within the 30 minutes time period applied here and under the used culture conditions.

In summary, these data show the differences in inflammatory protein expression and IL-1β release between the generated BMDCs and macrophages. Whereas ATP stimulation led to an enhancement of IL-1β secretion only in BMDCs, MSU did not influence the cytokine release. So, in DCs the IL-1β release is mainly triggered by ATP and not by uric acid, which probably activates other cellular responses.

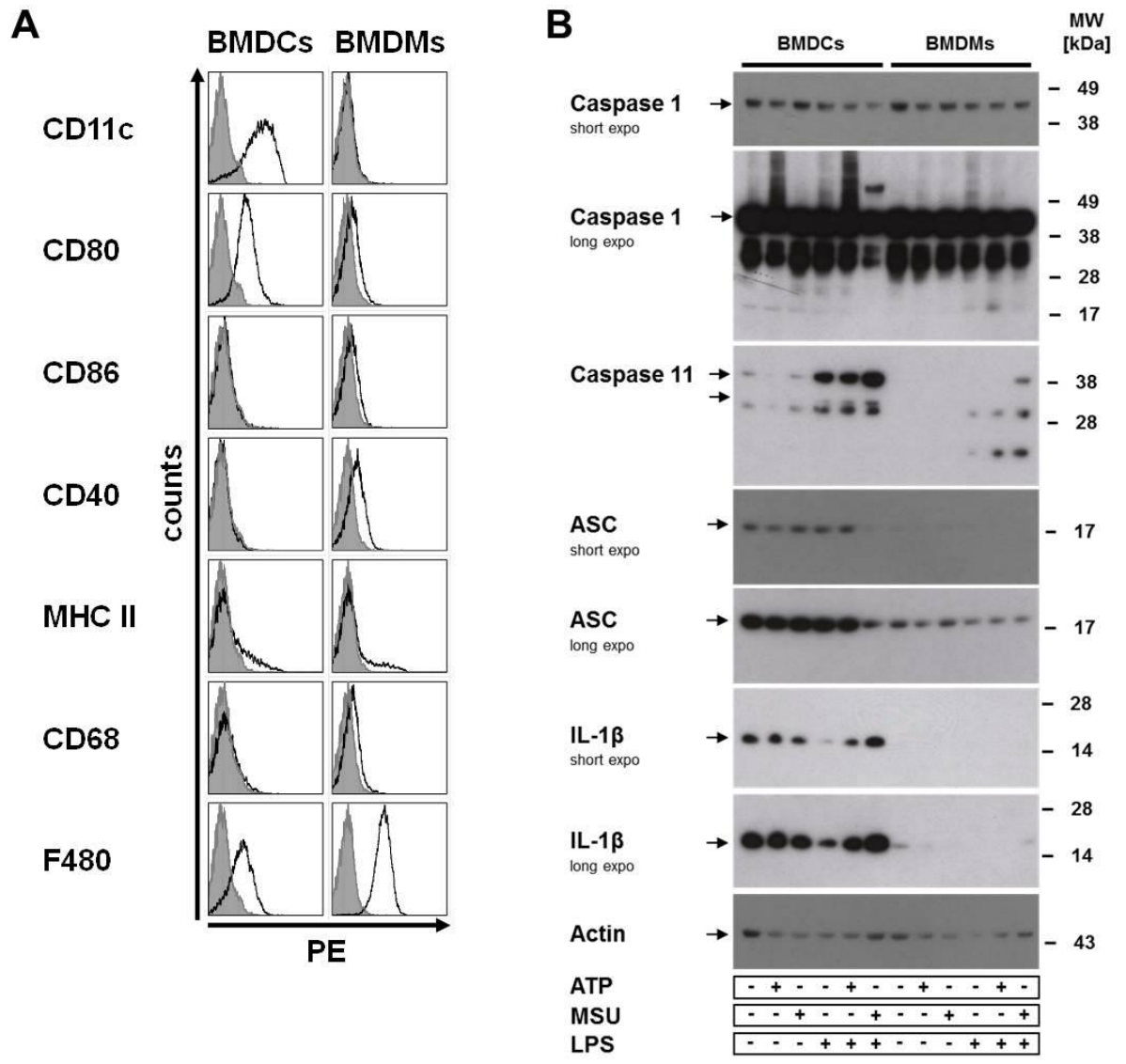

A
BMDCs
BMDMs
CD11c
CD80
CD86
CD40
MHC II
CD68
F480
counts
PE
B
BMDCs
BMDMs
MW [kDa]
Caspase 1 short expo
Caspase 1 long expo
Caspase 11
ASC short expo
ASC long expo
IL-1β short expo
IL-1β long expo
Actin
49
38
28
17
43
14
ATP
MSU
LPS
C
Fold of IL-1β secretion [relative units]
BMDCs
BMDMs
30
20
10
-
ATP
MSU

Figure 10

Figure 10: Influence of monosodium urate (MSU) or ATP stimulation on IL-1β release in bone marrow-derived primary DCs (BMDCs) and bone marrow-derived primary macrophages (BMDMs).
Bone marrow cells of C57 BL/6 mice were isolated. One part of the cells was differentiated with 30 µg/ml of M-CSF to generate macrophages and cultured in cell culture surface treated plates. The other part of the cells was cultured in non-surface treated plates with GM-CSF-containing media to obtain DCs. Cells were matured for six hours with 1 µg/ml LPS and stimulated with 300 µM ATP, or 5 µg/ml MSU for 30 minutes. (A) DC-, macrophage- and maturation- marker surface expression was analysed by flow cytometry. Corresponding isotype controls are shown in grey within the histograms. (B) Protein expression of the inflammatory molecules pro-IL-1β, Caspase-1, ASC and Caspase-11 was analysed by Western blotting, and β-actin served as an internal loading control. (C) The cytokine secretion was measured by ELISA. Data are representative of three independent experiments and are expressed as mean values ± SE.

3.1.5. The inflammasome activation in DCs is caspase- and potassium- but not calcium dependent.

Next, I wanted to test if the inflammasome activation depends on potassium gradient and on caspase activation. Thus, I performed experiments with mature DCs under high extracellular potassium conditions in combination with ionomycin and with the pan-caspase inhibitor zVAD-fmk. Upon treatment with those reagents, cells were stimulated with ATP, secretion of IL-1β was determined by ELISA, protein expression was analysed by Western Blot.

The IL-1β release after ATP stimulation of mature DCs is blocked by high extracellular potassium and by zVAD-fmk treatment (Figure 11 A). When caspases are inhibited, no major differences could be detected for the inflammatory molecules Caspase-1, Caspase-11, ASC and pro-IL-1β upon ATP stimulation. Treatment with zVAD-fmk of LPS-primed DCs inhibited Caspase-11 cleavage into the p20/p18 fragments in Western Blot analysis (Figure 11 B). In contrast to previous findings in macrophages (Gallin 1984; Rosati et al. 1986), blocking of the potassium efflux in cocktail-matured DCs inhibited the release of IL-1β. Opening of the calcium channels with ionomycin also showed no effect on IL-1β secretion. Ionomycin did not compensate the effect of blockage by high extracellular potassium (Figure 11 C). Furthermore, no effects on protein expression level for Caspase-1, Caspase-11, ASC, pro-IL-1β and IL-1β were observed after treatment with ionomycin in

combination with ATP and under high extracellular potassium conditions (Figure 11 D). These results confirm that the IL-1β release upon inflammasome activation is caspase as well as potassium dependent and show that in DCs it is independent on calcium.

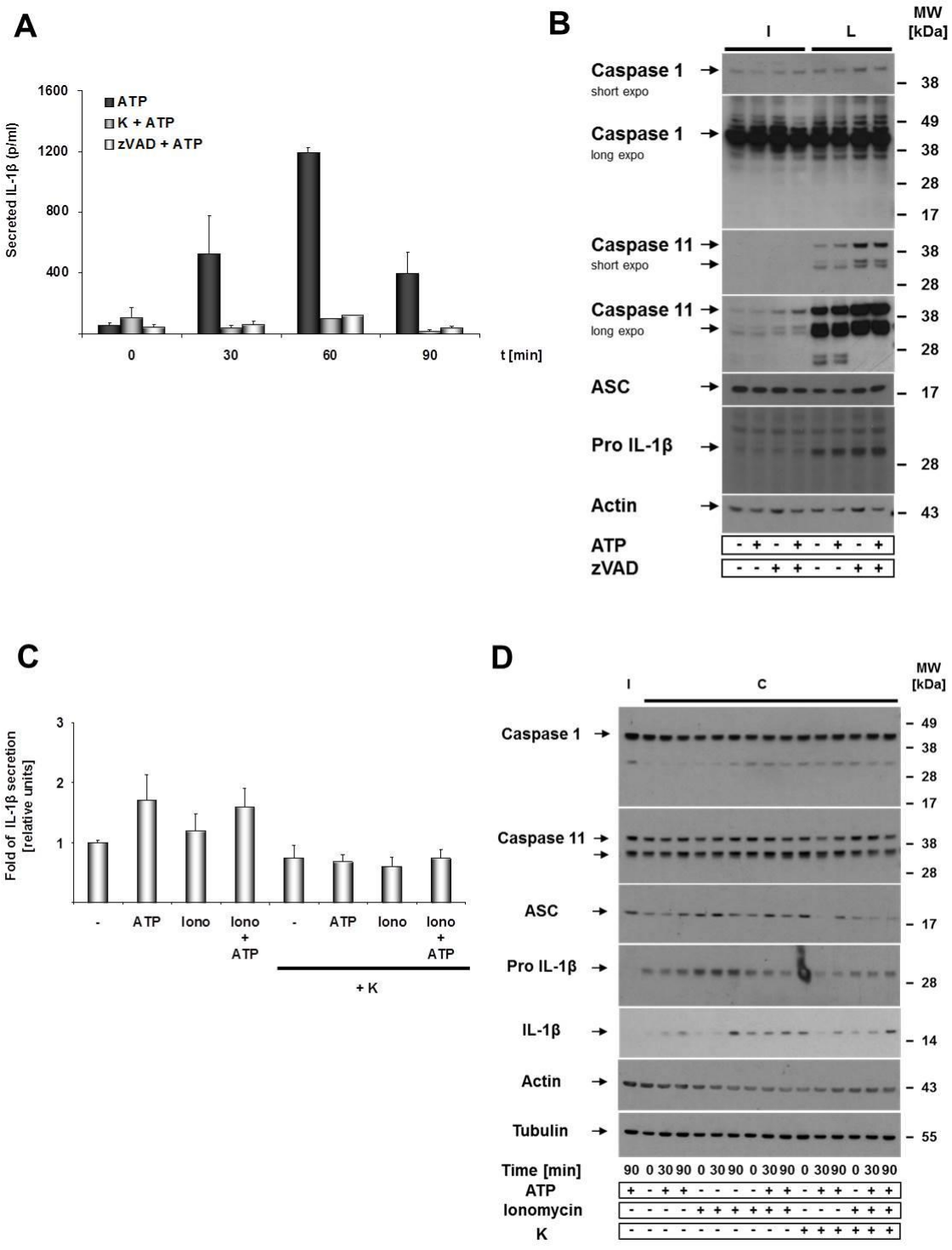


Figure 11

Figure 11: IL-1β release upon ATP stimulation depends on potassium gradient and caspase activation and not on calcium.
Bone marrow cells of C57BL/6 mice were isolated. Immature cells (I) were matured with 1 µg/ml LPS (L) or a stimulation-cocktail (C) containing TNF-α, IL-4, CD40L and PGE-2 for overnight. (A, B) Upon 20 min treatment with 40 µM zVAD-fmk cells were stimulated with 1 mM ATP for 0, 30, 60 or 90 minutes. (C, D) Mature DCs were treated with high extracellular potassium (130 mM) for 20 min, stimulated with 1 µg/ml ionomycin for 5 minutes and incubated with 1 mM ATP for another 30 minutes. Supernatants and cells were collected. (A, C) IL-1β cytokine secretion was measured by ELISA. Data are representative of three independent experiments and are expressed as mean values ± SE. (B, D) Protein expression of the inflammatory molecules pro-IL-1β, IL-1β, Caspase-1, ASC and Caspase-11 was analysed by Western blotting, and β-actin expression served as an internal loading control. Data are representative for three independent experiments.

3.1.6. Inhibition of gene translation diminished the expression level of pro-IL1β but did not block the ATP-induced IL-1β secretion.

Next, I investigated the effect of a blockage of the IL-1β secretion by inhibition of translation. Therefore, cycloheximide (CHX) was used 30 minutes prior to stimulation of mature DCs with ATP. Secretion of IL-1β was determined by ELISA, protein expression was analysed by Western Blot analysis. The ATP induced secretion of IL-1β could not be blocked by inhibition of translation by CHX treatment within the tested time period (Figure 12 A). The protein level of pro IL-1β diminished over time (Figure 12 B, lane 4, 5 and 6). The translational inhibition by CHX treatment was successfully working but did not yet show an effect on IL-1β secretion level after 60 minutes ATP stimulation. No significant differences could be detected in Western Blot analysis of caspase-1, caspase-11 and ASC expression (Figure 12 B).

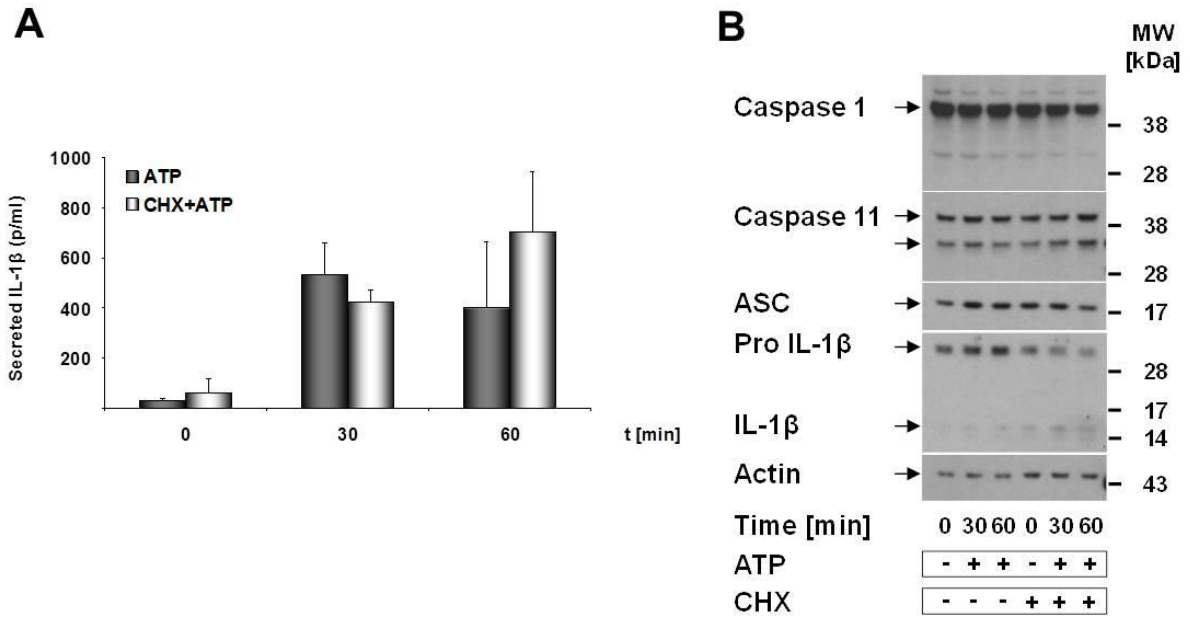


Figure 12

Figure 12: IL1-β release is unaffected in presence of cycloheximide (CHX) within DCs.
Bone marrow cells of C57BL/6 mice were isolated. Cells were matured overnight with a cocktail containing TNF-alpha, IL-4, CD40L and PGE-2. Upon 30 min treatment with 10 µg/ml cycloheximide, cells were stimulated with 1 mM ATP for another 30 minutes. Supernatants and cells were collected. (A) IL-1β cytokine secretion was measured by ELISA. Data are representative of three independent experiments and are expressed as mean values ± SE. (B) Protein expression of the inflammatory molecules pro-IL-1β, caspase-1, ASC and caspase-11 was analysed by Western blotting, β-actin expression served as an internal loading control. Data are representative for three independent experiments.

Summarizing the data, pro-IL-1β is constantly expressed in mature DCs and the transcription can be blocked by CHX treatment. Due to an accumulation of inflammatory proteins e.g. in microvesicles within the cells, the IL-1β secretion upon ATP stimulation is not yet affected by inhibition of translation. Probably, the incubation time was too short and the protein expression/storage levels within the cell are very high so that an effect of the translational inhibition was not yet visible on the protein level. For further analysis a flush out of the inflammatory proteins by ATP treatment before inhibition of translation could demonstrate the intracellular signal transduction regarding inflammasome activation and protein disposition.

The modulation of inflammatory complex formation by the expression of specific gene products represents another possibility to analyse inflammasome activation and protein disposition.

3.2. Interference within inflammatory signalling pathways by expression of dnASC in dendritic cells

3.2.1. Genetic modification of dendritic cells by lentiviral infection.

For the analysis of the role of inflammatory caspases in processing of IL-1β in DCs, I tried to interfere with the inflammasome complex via a dominant negative form of the adaptor molecule ASC in a lentiviral vector system approach. Therefore, a specific genetic modification of DCs for an uptake and expression of certain amounts of foreign DNA is crucial. So, I established a method for genetic modification of DCs. DCs are post mitotic and differentiated cells, which makes the commonly used technics like transfection not very promising. Thus, a lentiviral transduction system of the third generation was used, which was developed for genetic manipulation of CD34+ precursor cells (Dull et al. 1998; Oki et al. 2001). To test, if lentiviral particles sufficiently infect DCs, human and murine DCs were transduced with concentrated lentivirus titers. The efficiency of the infection was quantified by flow cytometric detection of the co-expressed eGFP. In Figure 13 A the FACS analysis of transduced murine DCs in comparison to untransduced DCs is shown. A clear shift of the whole population regarding the green fluorescence is seen and was confirmed in microscopic analysis, as well (Figure 13 B). Hence, DCs were genetically modified by lentiviral infection in a dose dependent manner. So, this method was suitable for generating genetic manipulated DCs.

Dendritic cells are natural sensors for invading bacteria and viruses as well as transformed cells. DC infection leads to DC maturation (Liechtenstein et al. 2013; Nasr et al. 2014; Schmidt et al. 2012; Toscano et al. 2010; Xiao et al. 2012; Zhang et al. 2013). Thus, I investigated if lentiviral infection of BMDCs in an early, immature stage led to cell maturation. Surface marker analysis by flow cytometry of

untransduced BMDCs versus empty control vector (CV) transduced cells, showed a clear upregulation of the maturation marker CD80 and increased expression of CD86 (Figure 13 C). This maturation effect due to lentiviral infection of DCs could be already accompanied by inflammasome activation. This has to be kept in mind for further discussion and demands a careful interpretation of out coming data.

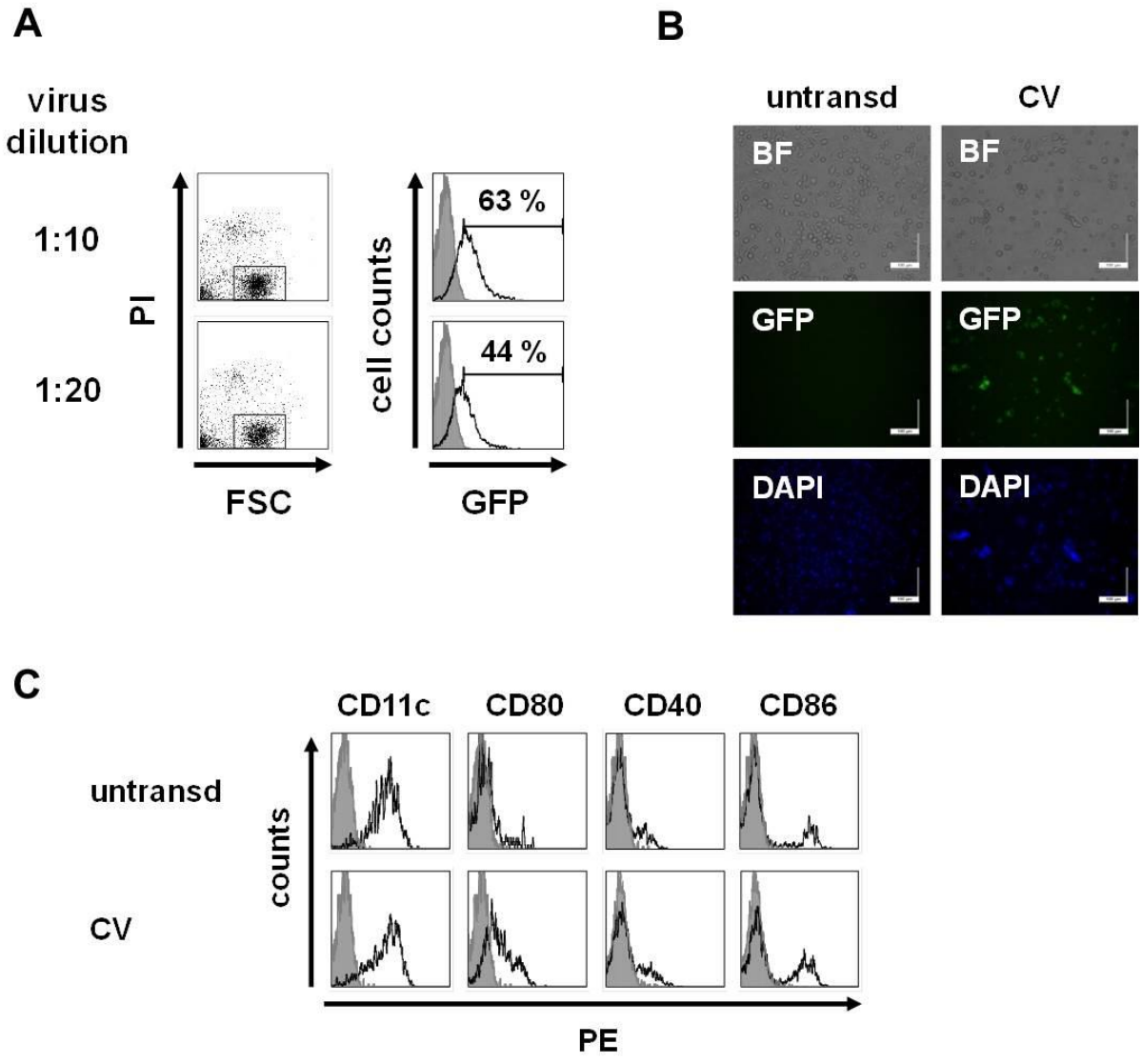


Figure 13

Figure 13: Lentivirus mediated genetic manipulation of DCs.
Bone marrow cells of C57BL/6 mice were isolated. BMDCs were infected with two concentrations of lentivirus containing a GFP encoding control vector (namely Tween). (A) GFP expression was analysed by flow cytometry. The dot plots for the size and PI positivity are shown for the infected cells on the left side. Untransduced cells are shown in grey within the corresponding histograms. (B) Microscopic pictures of untransduced (untransd) and control vector (CV) transduced BMDCs. Data are representative of three independent experiments. (C) DC and maturation marker expressed on the cell surface were assayed by flow cytometry. Corresponding isotype controls are shown in grey within the histograms. Data are representative of three independent experiments.

3.2.2. Interference with the inflammasome by over expression of dnASC in DCs.

Cellular, viral and microbial inhibitors (table 1, introduction) are used from some bacteria and viruses as natural way to overcome the immune system. In this work, I focused on the overexpression of a dominant-negative version of the adaptor molecule ASC, which is known to inhibit the NALP3 inflammasome signalling pathway. Analysis of the IL-1β secretion upon dnASC expression in different cell types served as indirect functional analysis for inflammasome inhibition. For over-expression of dnASC, I subcloned different dominant negative human and murine gene variants, which were subsequently verified by sequencing, into respective vectors. Generated viruses were used for infection of different cell types like THP-1, DC2.4, XS52 and primary BMDCs. The protein overexpression was assayed by Western blotting. THP-1 cells were successfully infected and expressed dnASC on protein level (Figure 14 A, B). Upon priming with MDP and LPS followed by stimulation with ATP, no secretion of IL-1β was detected in transduced THP-1 (Figure 14 C). Repetitions of functional experiments in the DC model cell lines DC2.4 (Figure 14 D) or XS52 (data not shown), indicate that these cells cannot be used as model for IL-1β secretion. Thus, functional experiments should be performed in primary cells as model for the analysis of inflammasome inhibition.

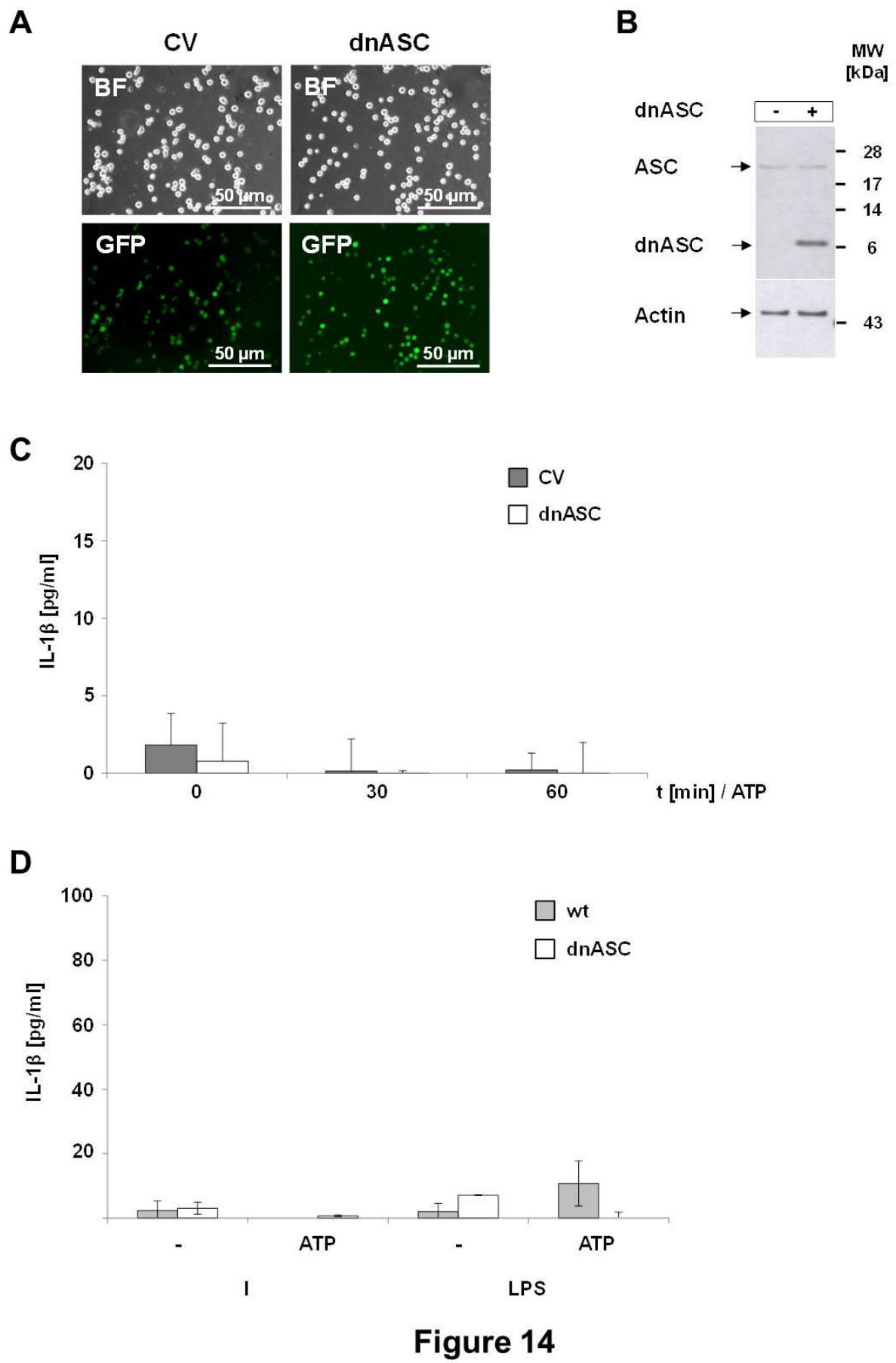

A
CV
dnASC
BF
BF
50 µm
50 µm
GFP
GFP
50 µm
50 µm
B
MW
[kDa]
dnASC
-
+
ASC
dnASC
Actin
28
17
14
6
43
C
IL-1β [pg/ml]
20
15
10
5
0
CV
dnASC
0
30
60
t [min] / ATP
D
IL-1β [pg/ml]
100
80
60
40
20
wt
dnASC
-
ATP
-
ATP
I
LPS

Figure 14

Figure 14: Functional analysis of dnASC in model cell lines.
THP-1 cells were cultured and infected/transduced with lentiviral supernatant containing the empty control vector (CV) Tween or the Tween–dnASC (dnASC) vector. (A) Transmission and fluorescence microscopic pictures of CV and dnASC transduced THP-1 cells. Data are representative of three independent experiments. Bars = 50 µm. (B) Protein expression of the adaptor molecule ASC was analysed by Western blot, and expression level of β-actin served as an internal loading control. Data are representative for three independent performed experiments. (C) THP-1 cells were primed with 1 µg/ml LPS and 2 µg/ml MDP for 3 hours and stimulated with 1 mM ATP for 30 and 60 minutes. IL-1β cytokine secretion was measured by ELISA. Data summarize two independent experiments and are expressed as mean values ± SE. (D) DC2.4 cells immature (I) or matured with 1 µg/ml LPS overnight (LPS) were stimulated with 1 mM ATP for 60 minutes. IL-1β cytokine secretion was measured by ELISA. Data summarize two independent experiments and are expressed as mean values ± SE.

3.2.3. Overexpression of dnASC via an inducible and selectable two-vector expression system.

The efficiency of lentiviral infection of DCs by virus is dose dependent, but could not be augmented from 60 % to 100 % (Koya et al. 2007; Masurier et al. 2007; Toscano et al. 2009; Zarei et al. 2002). Infection of DCs with a commonly used overexpression vector like Tween could lead to non-significant differences regarding the results due to contamination with uninfected cells (see also Figure 13). To circumvent this potential problem, another system with the possibility for selection and induction was used. This two-vector system, shown as a model in Figure 15 A, worked by double infection of cells with two viruses, namely the pFGEV16-Super-PGKHygro containing virus and the pF5xUAS-transgene-SV40-Puro containing virus. Upon selection of double infected cells with hygromycin and puromycin, the transgene transcription is activated only in the presence of 4-Hydroxy-Tamoxifen (4-HT). Generated lentiviral supernatants were used for transduction of different cell types. Already the production of virus containing supernatants encoding GFP led to a transgene expression in HEK293T cells, to a microscopically visible green fluorescence of those cells (Figure 15 B) indicating its effectiveness.

The overexpression of GFP and dnASC was first tested and functionally analysed in THP-1 cells. Briefly, THP-1 cells were primed with 1 µg/ml LPS and 0,5 µM PMA, followed by 1 mM ATP stimulation. Neither morphological changes nor an IL-1β release after dnASC transgene expression in those cells could be detected (data not shown) leaving functional data still open. So the DC - model cell lines tested here are not suitable for functional analysis of inflammasome mediated signalling pathways.

Next, I infected primary dendritic cells with the control- (GFP) and the Gev16 virus. If BMDCs were not selected with puromycin and hygromycin, only 1 % of the cells showed positivity for green fluorescence (Figure 15 D). The selection enhanced the amount of GFP positive cells to 4 % but also shifted the whole cell population into PI positivity indicating cell death (see FACS dot plots in Figure 15 D). In 5 repetitive experiments nearly no cells survived the selection (Figure 15 E). Thus, the infection of BMDCs with two viruses was limited and is not suitable for further functional analysis. The two-vector system nicely working in melanoma and other model cell lines (Geserick et al. 2009) could not be established for infection of those primary immune cells.

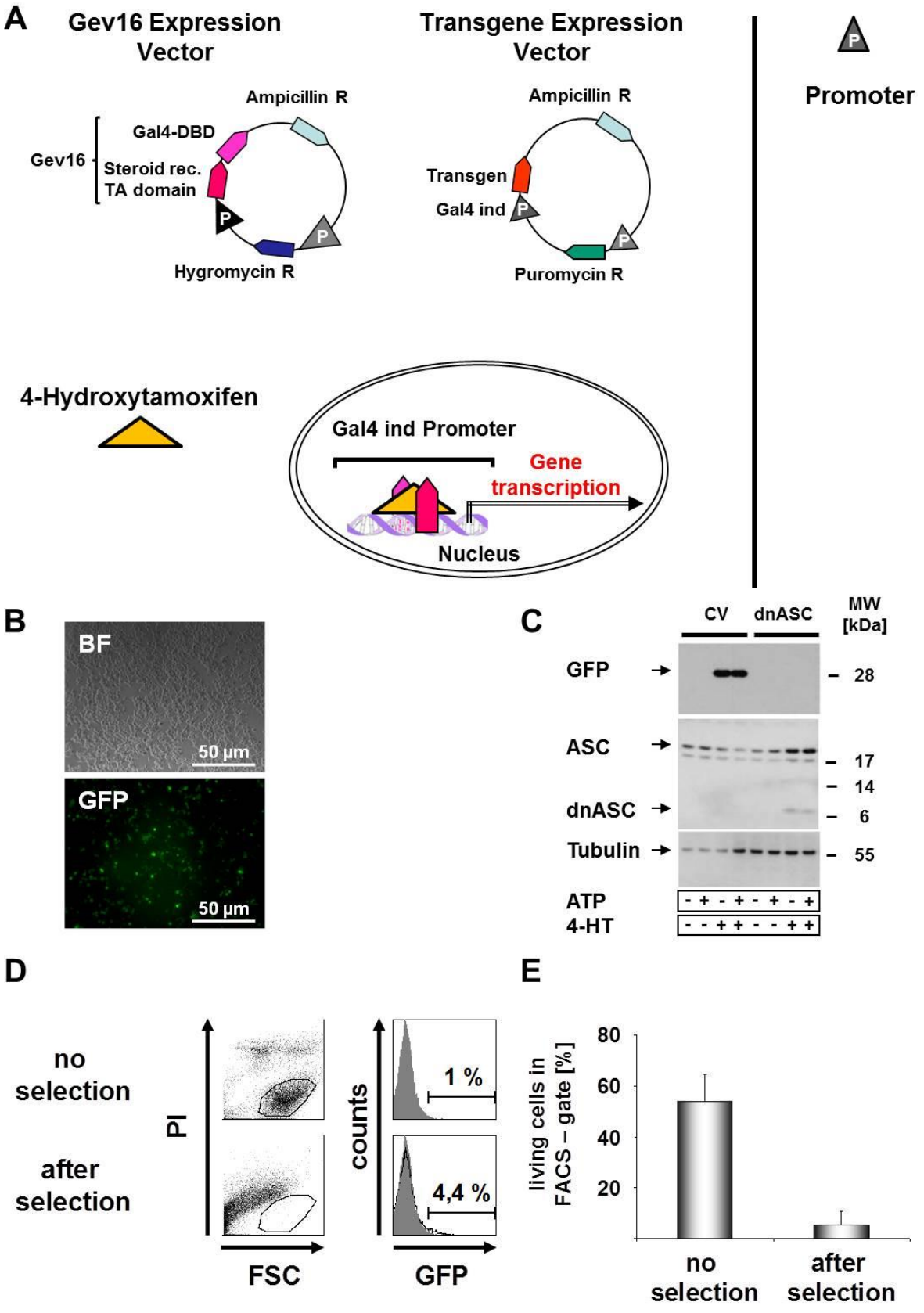

A
Gev16 Expression Vector
Ampicillin R
Gal4-DBD
Gev16
Steroid rec.
TA domain
P
P
Hygromycin R
Transgene Expression Vector
Ampicillin R
Transgen
Gal4 ind
P
P
Puromycin R
P
Promoter
4-Hydroxytamoxifen
Gal4 ind Promoter
Gene transcription
Nucleus
B
BF
50 µm
GFP
50 µm
C
CV
dnASC
MW [kDa]
GFP
28
ASC
17
14
dnASC
6
Tubulin
55
ATP
4-HT
- + - + - + - +
- - + + - - + +
D
no selection
after selection
PI
FSC
counts
GFP
1 %
4,4 %
E
living cells in FACS – gate [%]
80
60
40
20
no selection
after selection

Figure 15

Figure 15: Cloning of dnASC into a selectable two-vector (two-virus) system. Cells were double infected with a Gev16 expressing (Gev16 expression vector) hygromycin selectable virus and the puromycin selectable transgene expression vector encoding virus. Only in presence of 4-Hydroxy-Tamoxifen (4-HT) gene transcription is activated in double infected cells. (A upper part) Depicted is a scheme of the map of the Gev16 expression vector and the transgene expression vector. (A lower part) Scheme of gene transcription induction by 4-HT. (B) The production of control vector virus in HEK293T cells led to a visible expression of the GFP shown by fluorescence microscopy; bars = 50 µm. Shown is one out of five representative experiments. (C) Protein expression in selected GEV16 expressing THP-1 cells upon 4-HT (100 nM) induction; dnASC and GFP was analysed by Western blotting, β-tubulin expression served as internal loading control. (D) Cytometric analysis of cell state by forward scatter, PI positivity and GFP expression of virally infected primary BMDCs with and without selection. (E) Percentage of surviving BMDCs after two-virus infection based on the FACS data; n=5.

3.2.4. Overexpression of dnASC via an inducible and selectable all-in-one vector system.

Due to difficulties regarding the infection of dendritic cells with two different lentiviruses, the respective molecules GFP (as control) and dnASC were subcloned into an “all in one” pF 5x UAS MCS SV40 puro GEV16 W lentiviral vector kindly provided by Dr. John Silke, which is also 4-HT inducible and puromycin selectable (Figure 16 A). Successfully subcloned constructs were analysed by specific restriction and subsequently verified by sequencing (data not shown). The sequence verified constructs were used to generate lentiviral supernatants for transduction of different cell types. The overexpression of GFP and different ASC proteins was first tested in THP-1 cells and analysed upon selection with puromycin and treatment with different concentrations of 4-HT for 24h (Figure 16 B, C, D). The selection with puromycin followed by 4-HT induction of transgene expression in infected THP-1 cells augmented GFP expressing cell number to 91.9 % (Figure 16 B). If 4-HT was omitted, GFP expression was below 1 %. So, the inducible system is nicely working in THP-1 cells, no leakiness was observed.

For the analysis of functional consequences of impaired ASC function, primary human keratinocytes as another cell type were tested in comparison to THP-1 cells (Figure 16 C). Keratinocytes are cells of the skin which functions as the protective barrier against pathogens, physical and chemical harms. UVB light exposure of keratinocytes leads to secretion of the proinflammatory cytokines IL-1β and IL-18, which depends on inflammasome activation (NALP1, NALP3, ASC, Caspase-1) (Feldmeyer et al. 2007). All components of the inflammasome are released together with the cytokines. Yet, the mechanisms involved are still poorly defined. For functional analysis the dnASC, infected, selected and induced primary human keratinocytes were treated with 100 mJ/cm^2 UVB light.

Feldmeyer and Co-workers showed that a si-RNA mediated silencing of ASC lead to dramatic suppression of IL-1β release following UVB exposure in keratinocytes, which was confirmed by the here used dnASC overexpression model system (Feldmeyer et al. 2007; Marionnet et al. 1997; Park et al. 2006; Skiba et al. 2005; Wiswedel et al. 2007). UVB stimulation enhanced the IL-1β secretion in control vector infected keratinocytes; however, dnASC expression diminished the IL-1β secretion comparable to the non-infected control (Figure 16 D). Experiments were performed in 4 different donors and indicate that a viral infection lead to activation of the inflammasome in keratinocytes. Virally-mediated overexpression of dnASC in keratinocytes inhibits the secretion of IL-1β upon UVB stimulation, arguing for comparative effects like shown for the si-RNA mediated knockdown approach. Due to the generated functional data, dnASC overexpression can finally be tested in primary DCs.

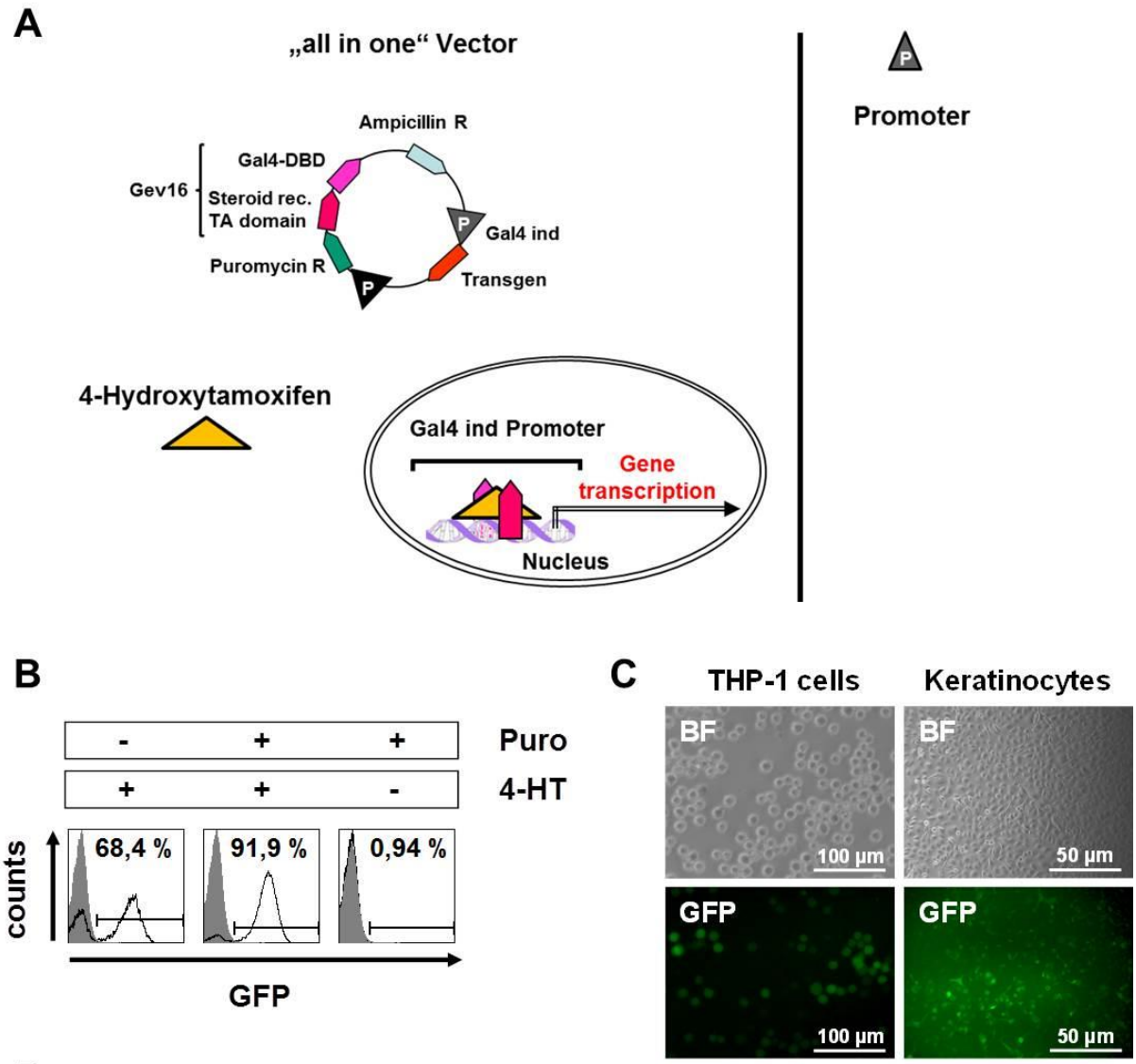

A
„all in one“ Vector
Ampicillin R
Gal4-DBD
Gev16
Steroid rec.
TA domain
Gal4 ind
Puromycin R
Transgen
P
Promoter
4-Hydroxytamoxifen
Gal4 ind Promoter
Gene
transcription
Nucleus
B
-
+
+
Puro
+
+
-
4-HT
68,4 %
91,9 %
0,94 %
counts
GFP
C
THP-1 cells
Keratinocytes
BF
BF
100 µm
50 µm
GFP
GFP
100 µm
50 µm
D
Normal Light
UVB [100 mJ/cm²]
Fold of IL-1β secretion
[relative units]
30
25
20
15
10
5
nk
CV
dnASC

Figure 16

Figure 16: Cloning of dnASC into a selectable "all-in-one"-vector and test of protein expression after 4-HT treatment in different cell lines.
Briefly, the corresponding mouse dnASC, human dnASC or GFP genes were subcloned into the pF 5x UAS MCS SV40 puro GEV16 W lentiviral vector ("all in one" vector). (A) Map of the inducible, puromycin-selectable lentiviral transgene expression vector. Transgene transcription is induced after 4-HT-treatment. The expression of the GFP expressing control construct after 4-HT induction in selected THP-1 cells was analysed by FACS (B) and by fluorescence microscopy (C, left panel). The GFP expression was also tested in infected primary keratinocytes of different donors (C, right panel). Data of one out of three independent experiments are shown; bars as indicated. As functional analysis of dnASC-expression the IL-1β secretion was analysed after UVB irradiation of primary keratinocytes by ELISA, n=3 (D).

DCs were infected with lentivirus carrying the "all in one" vector and analysed in parallel to the functional experiments performed within keratinocytes. Upon selection with puromycin 37 % of control vector (GFP) infected cells and 20 % of dnASC infected cells were detected in the FACS gate for living cells (Figure 17 A). However only 11 % of the control vector infected DCs were successfully transfected as shown by GFP expression, which is summarized for three independently performed experiments in Figure 17 C. So, not all infected cells were expressing detectable amounts of the transgene. Upon 6 h maturation with 1 µg/ml LPS and 30 minutes stimulation with 1 mM ATP, protein expression and IL-1β secretion was analysed (Figure 17 B, D). I detected GFP and dnASC after 4-HT mediated gene induction in infected cells (Figure 17 B). Stimulation with ATP did not show any effects on GFP or dnASC protein expression nor on IL-1β secretion (Figure 17 D). For the impact on the inflammatory IL-1β secretion, the inhibition of the inflammasome by dnASC in DCs still remained open. The diminished survival of transgene expressing cells (10 % to 20 %), the survival of non-transgene expressing cells as well as all the dead cells within cell culture observed in FACS analysis (Figure 17 A) during functional experiments are possible explanations why there is no inhibitory effect seen on IL-1β secretion by dnASC. A counter regulation within the cell and the self-oligomerization of dnASC molecules (also called ASC^{PYD} filaments), which leads to a diminished number of available proteins (Bryan et al. 2009; de 2009; Liepinsh et al. 2003; Lu et al. 2014), could also explain the observed results. Further

experiments in DCs should be performed after sorting of living and transgene expressing cells.

Informative, by testing the “all in one” GFP expressing construct in primary keratinocytes, I saw green fluorescence also in the non-4-HT induced sample, which was confirmed by Western Blot analysis (Figure 17 E first panel, F). This finding depicts a protein expression independent on 4-HT induction in these primary cells, which was not detected in THP-1 cells (Figure 16 B). Duo to a leakiness of the system within primary keratinocytes and generated negative results in THP-1 cells, further experiments regarding inflammatory signalling pathways in this work were performed in primary DCs.

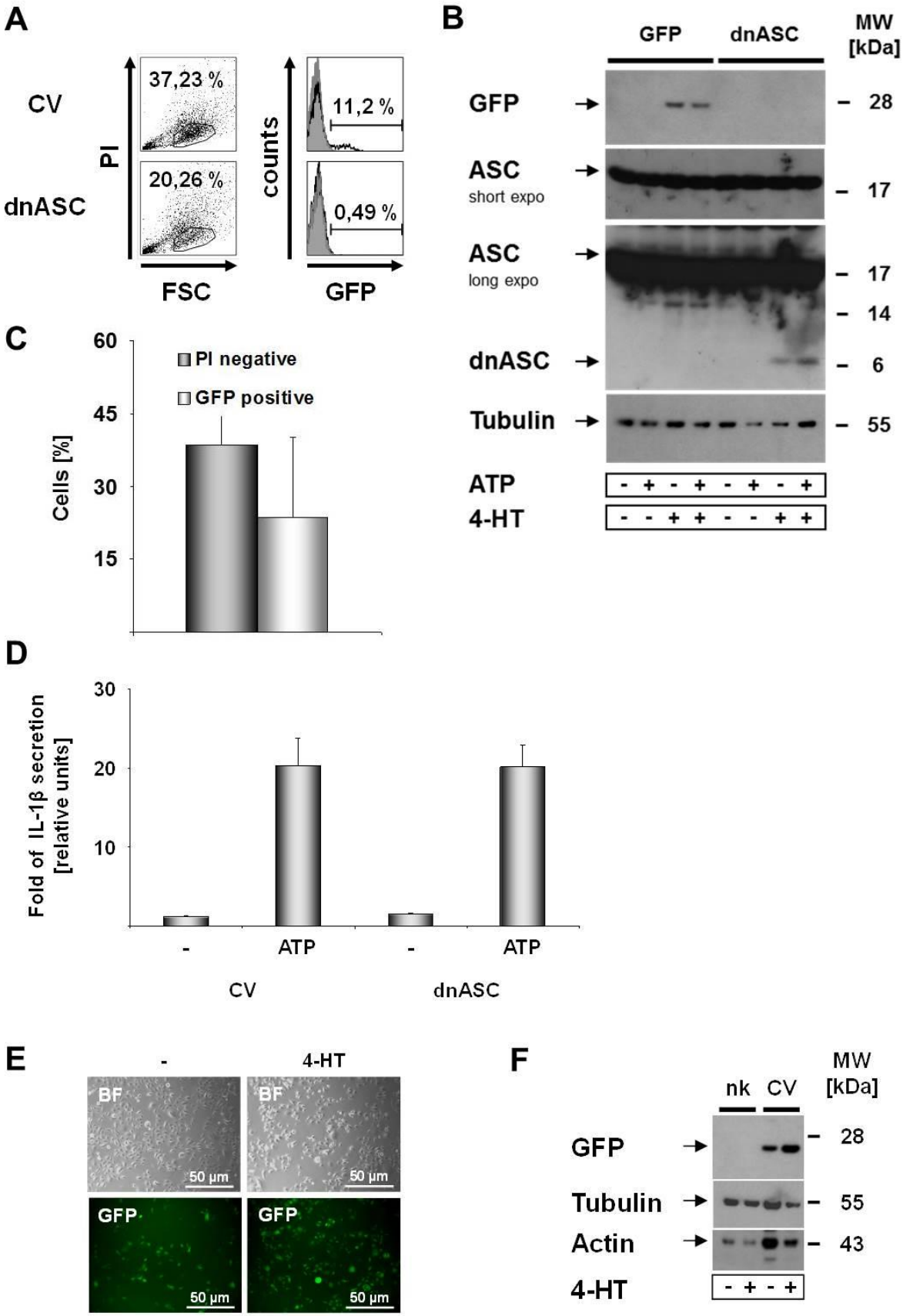

A
CV
dnASC
37,23 %
20,26 %
PI
FSC
11,2 %
0,49 %
counts
GFP
B
GFP
dnASC
MW [kDa]
GFP
ASC short expo
ASC long expo
dnASC
Tubulin
ATP
4-HT
28
17
17
14
6
55
C
Cells [%]
PI negative
GFP positive
60
45
30
15
D
Fold of IL-1β secretion [relative units]
30
20
10
-
ATP
-
ATP
CV
dnASC
E
-
4-HT
BF
GFP
50 µm
F
nk
CV
MW [kDa]
GFP
Tubulin
Actin
4-HT
28
55
43

Figure 17

Figure 17: Expression of dnASC after 4-HT stimulation in infected and selected DCs and primary keratinocytes.
DCs were virally infected using the “all-in-one” construct with dnASC and the GFP only expressing vector served as negative control (CV). (A) FACS analysis of cell state and GFP expression of virally infected primary DCs with and without selection. (B) Protein expression upon 4-HT-mediated induction of ASC, dnASC and GFP was analysed by Western blotting, and expression level of β-tubulin served as internal loading control. (C) Summary for cell surviving and GFP expression of DCs after control-virus infection, selection and induction of three independent experiments is indicated in a diagram. (D) The secretion of the inflammatory cytokine IL-1β upon dnASC or GFP expression after ATP stimulation was determined by ELISA. The summary of two independently performed experiments is shown. (E, F) GFP expression upon 4-HT-mediated induction virally infected in primary keratinocytes (donors 11 and 12) with dnASC or CV vector was analysed by fluorescence microscopy (E) and by Western-blot analysis (F). One of two representative experiments is shown (donor 11).

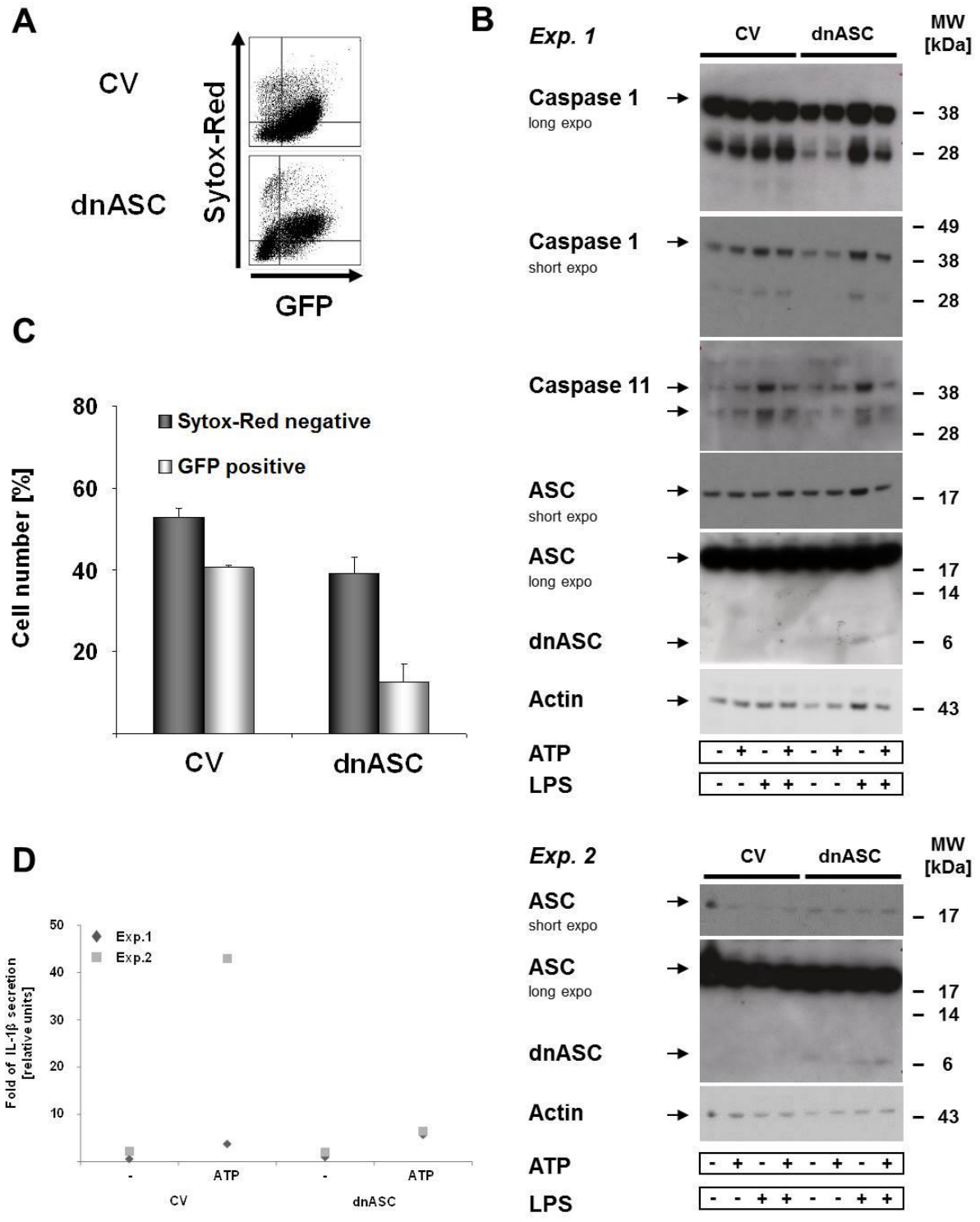
3.2.5. Commonly used overexpression followed by cell sorting.

Another possibility to overcome problems for the expression and the leakiness is to overexpress the dnASC with the Tween vector and to perform the experiments in positive, living, FACS-sorted cells.

Virally infected BMDCs were stained with a dead cell marker (Sytox-red) and were sorted. GFP positive and Sytox-red negative cells were collected and further used for the analysis of the impact of dnASC expression e.g. on IL-1β release. In all sorting experiments a whole shift of the cell population into Sytox-red positivity was seen in dnASC transduced samples (Figure 18 A), summarized in a diagram in Figure 18 C for two independent experiments. The expression of dnASC led to a severe cell death in primary dendritic cells, which limited further analysis of the inflammatory signalling cascade. Only 10 % of dnASC transduced cells were GFP-positive and viable and could be used for further experiments upon FACS-mediated cell sorting (Figure 18 C). After sorting and with a purity of 72 % to 77 %, in 2 out of 4 independent experiments the cell number was sufficient to perform functional analysis. Cells were matured with LPS and stimulated with ATP for 30 minutes. Supernatants and cells were collected; IL-1β secretion was analysed by ELISA, and protein expression was determined by Western Blot.

The ATP-induced IL-1β secretion could be blocked upon dnASC expression in primary dendritic cells only in one out of two independent experiments (Figure 18 D), because in the second experiment the ATP stimulation did not work and enhanced the IL-1β secretion in the control vector transduced cells. No differences in expression levels for Caspase-1, Caspase-11 and ASC could be seen upon LPS maturation and ATP stimulation (Figure 18 B). The irregularities seen in the blots are due to differences in loading, shown by β-actin detection. The expression of the dnASC molecule in infected DCs was detected by Western Blot analysis. However, the amount of detected dnASC compared to full length ASC was very low in these cells (Figure 18 B, compare also with Figure 17 B). Based on the functional data in one out of two experiments and on the fact, that the polyclonal antibody was developed against full length ASC (and not only the PYD domain), the distribution of protein expression seen in the analysis possibly does not represent the intracellular situation.

Furthermore, the non-inducible dnASC is expressed over six days upon transduction during the whole experimental procedure, probably partially blocking the inflammasome signalling pathways and so mimicking a strong infection of the DCs. In most of the dnASC expressing DCs, inhibition of the inflammasome then possibly activated cell death pathways. The small amount of surviving cells perhaps counter-regulate this dnASC-mediated at that time unknown cell death pathway.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CV
dnASC
Sytox-Red
GFP
B
Exp. 1
CV
dnASC
MW [kDa]
Caspase 1
long expo
38
28
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short expo
49
38
28
Caspase 11
38
28
ASC
short expo
17
ASC
long expo
17
14
dnASC
6
Actin
43
ATP
LPS
C
Cell number [%]
80
60
40
20
Sytox-Red negative
GFP positive
CV
dnASC
D
Fold of IL-1β secretion [relative units]
50
40
30
20
10
Exp.1
Exp.2
-
ATP
-
ATP
CV
dnASC
Exp. 2
CV
dnASC
MW [kDa]
ASC
short expo
17
ASC
long expo
17
14
dnASC
6
Actin
43
ATP
LPS

Figure 18

Figure 18: DCs were infected with dnASC and functionally tested upon sorting for GFP and viability.
DCs transduced with viral titers using the Tween construct with dnASC (dnASC) and the empty vector served as negative control (CV). Cells were FACS-sorted for SytoxRed negativity (living cells) and GFP positivity (gene expression). Sorted DCs were matured for 6 h with 1 µg/ml LPS and stimulated with 1 mM ATP for 30 minutes. Supernatants and cells were collected and further analysed. (A) FACS analysis of cell morphology and GFP expression of transduced primary DCs for cell sorting, the population in the lower right quadrant was sorted. (B, Exp.1 and Exp. 2) Protein expression of Caspase-1, Caspase-11 and ASC upon ATP stimulation of LPS-matured and immatured DCs, expressing dnASC and control vector, respectively, was analysed by Western blotting. Expression level of β-actin served as internal control for equal protein loading. (C) Summary of DC cell survival and GFP expression after viral infection before sorting of 2 independent experiments is shown. (D) The secretion of the inflammatory cytokine IL-1β upon dnASC or control vector expression and ATP stimulation was determined by ELISA. The analysis of two independent experiments is shown.

Summarizing the results, only in one out of two experiments the IL-1β secretion was partially blocked by expression of dnASC in DCs. So, the function remains still unclear; data need to be verified by repetition experiments. Beside activation of the inflammatory signalling cascade by cellular, viral and microbial PAMPs, DCs depict characteristic gene expression pattern relevant for optimal immune responses and concomitant triggering of cell death signalling. Especially the cytokine panel after cell-cell interaction determines the direction of immune response and TC differentiation. To elucidate the impact of inflammatory cytokines, especially IL-1β, on TC priming and their role in the CD4+ TC-DC *in vitro* interaction model system, the cytokine panel, TC proliferation and cell death after cell-cell interaction was analysed.

3.3. Impact of inflammatory cytokines especially IL-1β on T-cell priming

3.3.1. The CD4+ TC-DC interaction model and the impact of IL-1β secretion on T-cell proliferation.

To test the impact of IL-1β during DC-TC interaction, an immunological synapse was simulated *in vitro*. CD4+ TCs of the OT2 mouse model were isolated from spleen, phenotyped and cultured with unloaded or ovalbumin-loaded mature DCs. TCs showed CD4 but no CD8 and CD28 positivity (Figure 19 A) and formed typical interaction clusters with ovalbumin-loaded DCs (Figure 19 B). Upon 60 hours cell-cell interaction *in vitro*, TC proliferation was determined by monitoring CFSE content and IL-2 secretion. TC proliferation resulted in IL-2 secretion (Figure 19 D) and up to four cell divisions were detected in CFSE-based FACS analysis 60 hours after TC-DC conjugation (Figure 19 C). Stimulation of TCs with 100 pg/ml recombinant IL-1β protein did not lead to cell proliferation (Figure 19 C) indicating that the cytokine alone is not sufficient and a cell-cell contact probably needed for TC activation.

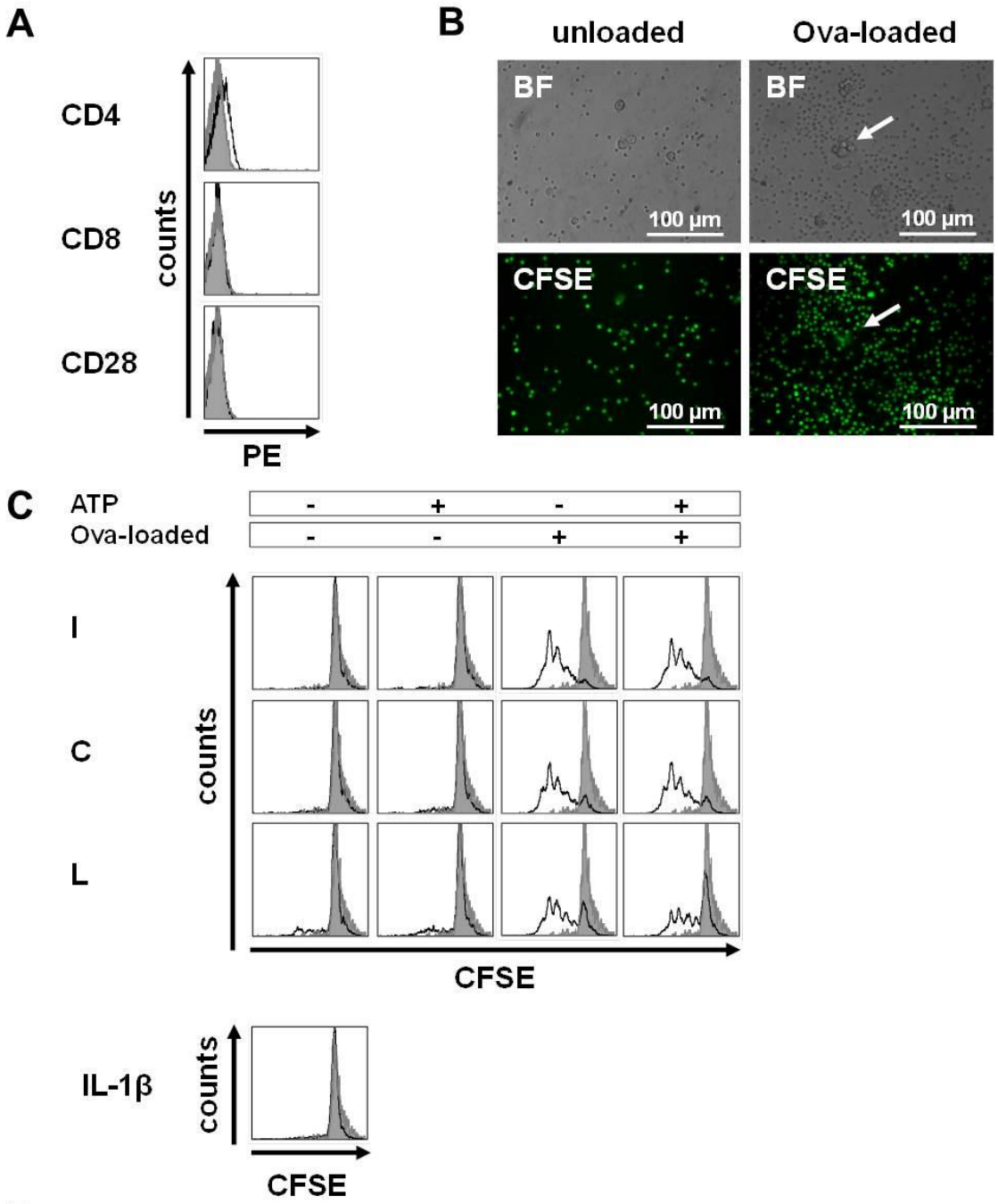

A
CD4
CD8
CD28
counts
PE
B
unloaded
Ova-loaded
BF
BF
100 µm
100 µm
CFSE
CFSE
100 µm
100 µm
C
ATP
Ova-loaded
- + - +
- - + +
I
C
L
counts
CFSE
IL-1β
counts
CFSE
D
Fold of IL-2 secretion [relative units]
120
80
40
Unloaded DC + TC
Ova loaded DC + TC

Figure 19

Figure 19: The DC-TC interaction model.
TCs were isolated from spleen from the OT2 mouse strain via MACS-separation. DCs were generated, matured and loaded with 250 µg/ml of ovalbumin for 6 h. Then they were stimulated for 30 minutes with 1 mM ATP and cocultured with CFSE labelled TCs for 60 hours. (A) TC surface marker expression was analysed by flow cytometry. The *in vitro* DC-TC interaction was analysed microscopically (B) and by FACS (C). Shown is one out of three representative experiments. (C) One part of the TCs was stimulated with recombinant 100 pg/ml IL-1β proteins. (D) The secretion of the cytokine IL-2 was determined by ELISA. The analysis of three independent experiments is shown.

3.3.2. Investigation on the direction of TC response upon DC-TC interaction.

Furthermore, to determine the direction of the immune response in the used model system a cytokine panel after interaction was dissected. Upon DC-TC interaction TNF-α, IL-2, IL-4, IL-10, GM-CSF, IFN-γ, G-CSF and M-CSF were detected in three independent experiments (Figure 20 A). Thus, the direction of the immune response seems to be ambiguous into Th1 (IL-2, IFN-γ, IL-12, TNF-β), Th2 (IL-4, IL-10, IL-5, IL-6, IL-13) and Th22 (TNF-α, IL-22). In the first two experiments, G-CSF was secreted after Ova-specific DC-TC interaction whereas no M-CSF could be detected (Figure 20 A*). To test the impact of released IL-1β (and IL-18) after ATP stimulation on TC proliferation, the supernatant of DCs was used in parallel to Ova-Peptide loaded DCs. No proliferation of TCs could be detected if supernatants of DCs were used, which was also observed when employing 100 pg/ml recombinant IL-1β protein (Figure 20 B). These data confirm that IL-1β and other secreted cytokines alone are not sufficient to initiate TC proliferation and support the hypothesis that cell-cell contact is required. Furthermore, the cytokines IL-2, TNF-α, IL-2, IL-4, IL-10, GM-CSF, IFN-γ, G-CSF and M-CSF detected in the used DC- CD4+ -TC interaction model system indicate that the cytokine network is more complex. The DC maturation by LPS combined with $P2X_7$ stimulation by ATP does not lead to one specific/directed TC response. The immune reaction and direction probably depends on the infection background especially on the amount and composition of pathogen molecules.

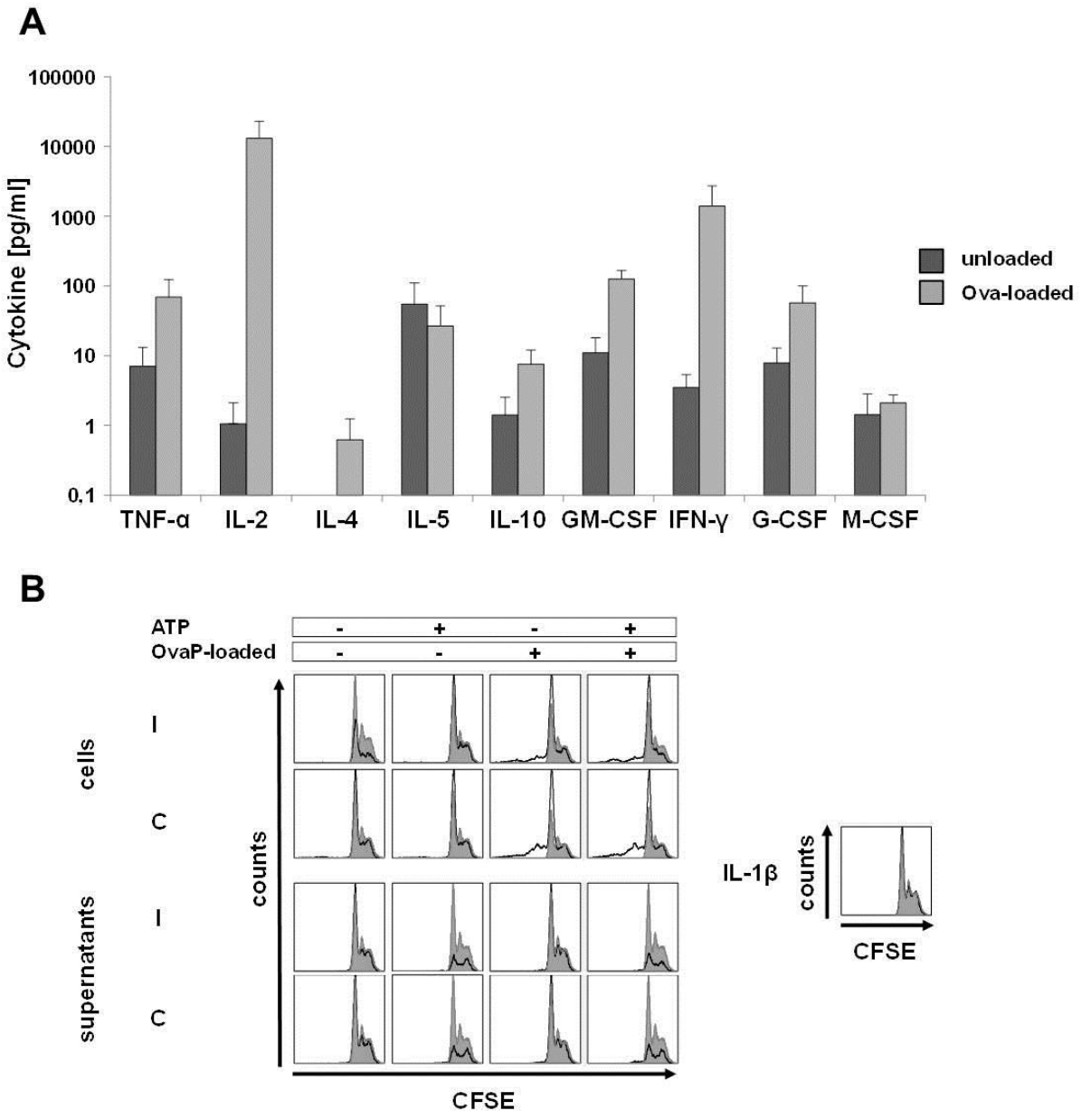

A
100000
10000
1000
100
10
1
0.1
Cytokine [pg/ml]
unloaded
Ova-loaded
TNF-α
IL-2
IL-4
IL-5
IL-10
GM-CSF
IFN-γ
G-CSF
M-CSF
B
ATP
OvaP-loaded
cells
I
C
supernatants
I
C
counts
CFSE
IL-1β
counts
CFSE

Figure 20

Figure 20: Analysis of the impact of IL-1β on $CD4^+$ TC proliferation.
TCs were isolated from spleen from OT2 mice via MACS-separation. DCs were generated, matured and loaded with 1 µg/ml Ova-peptide for 6 hours and stimulated for 30 minutes with 1 mM ATP. Supernatants and cells were collected and cocultured separately with CFSE-labelled TCs. TCs were stimulated with recombinant 100 pg/ml IL-1 β proteins. Upon 60 hours interaction, supernatants were collected and cells were analysed by FACS. The expression of nine cytokines (TNF-α, IL-2, IL-4, IL-5, IL-10, IFN-γ, GM-CSF, G-CSF, M-CSF) was determined two to three times with similar results grouped in A. (B) The *in vitro* DC-TC interaction was analysed by measuring TC proliferation using CFSE staining and FACS. Shown is one out of three representative experiments.

Upon activation of the immune response, the clearance of infected cells including DCs that acted as APCs needs to be guaranteed and additionally stimulates the activation of the immune system via the release of the pathogens, cytokines and PAMPs from dying cells. The provoked DC death can be initialized by intrinsic, extrinsic and TC-mediated signals. Interestingly, an uptake of apoptotic DCs by viable DCs can also induce tolerance via priming of antigen specific regulatory TCs. The characterisation of apoptotic signalling pathways in the used DC model system is another important part, which needs to be examined. Due to the diversity of DC death, the analysis of only one extrinsic signalling-mediated pathway namely the CD95L-mediated cell death pathway was investigated in this thesis.

3.4. Characterisation of apoptotic signalling pathways in dendritic cells

Based on previous findings, that c-FLIP was described as one key molecule inhibiting the CD95L-mediated signalling pathway probably by preventing caspase-8 activation at the DISC, the role of cFLIP in our murine DC model system was investigated. Therefore, the expression and function of the cFLIP protein under non-infected and siRNA-mediated knockdown conditions were analysed for different matured DCs. Furthermore, the role of caspases and RIPK1 was investigated for dissection of the observed CD95L-induced cell death.

3.4.1. Time-dependent regulation of cFLIP and sensitivity to CD95L-induced apoptosis upon activation of BMDCs.

First of all, intracellular proteins involved in the regulation of sensitivity and resistance to death ligands especially CD95L were analysed. To test the functional impact of cFLIP expression on CD95L-induced cell death, primary murine bone-marrow derived DCs (BMDC) were studied during different time points of DC maturation. Immature BMDCs were stimulated with LPS for 30, 60, 180, 360 minutes and overnight and relative cFLIP and caspase-8 expression was first determined by real-time quantitative PCR analysis (RT-PCR). LPS stimulation resulted in a rapid increase of cFLIP mRNA expression in a time-dependent manner, whereas mRNA levels of caspase-8 remained unchanged (Figure 21 A). In order to verify these results at the protein level, cFLIP protein was measured by Western blotting at the same time points. In accordance with the mRNA data, these experiments clearly demonstrated that the increase of cFLIP mRNA correlated with cFLIP protein expression (Figure 21 B). Caspase-8 mRNA and protein expression as well as FADD protein expression remained unaltered upon LPS stimulation (Figure 21 A, B). In summary, the DC maturation status correlated with expression levels of cFLIP on mRNA and protein level, the expression of Caspase-8 and FADD remained unaffected by maturation processes.

LPS-treated BMDCs were also analysed for changes in cell surface expression of maturation markers by FACS analysis. Our data demonstrated an increased DC maturation within 3-6 hours of LPS stimulation (Figure 21 C).

Next, I hypothesized that in murine DCs the maturation-dependent death ligand sensitivity inversely correlates with cFLIP and CD95 receptor surface expression as described in previous reports for human DCs (Leverkus et al. 2000b; McLellan et al. 2000). For verification of these findings, I investigated the CD95 surface expression and the CD95L-sensitivity in murine DCs at different maturation stages. However, CD95 surface expression was below detection level in the different analysed BMDCs populations, at least with the antibody used in our study (data not shown). Additionally, I tested the different murine DC populations for their sensitivity to CD95L-induced cell death. Indeed, compared to immature DCs, mature DCs were more resistant to CD95L-induced cell death demonstrated by reduction of hypodiploid nuclei (Figure 21 D). Consistent with a decreased sensitivity to apoptotic cell death, I observed delayed and inhibited processing of caspase-8 within the mature DC population. In contrast, caspase-8 cleavage in the immature DC population was detected after 15 minutes of stimulation with CD95L (Figure 21 E). Taken together our data suggest that cFLIP expression in DCs is rapidly upregulated by LPS and seems to precede DC maturation, at least when assayed by surface expression of maturation markers, and that the increased levels of cFLIP by LPS maturation confered resistance to CD95L-induced apoptotic cell death in BMDCs. According to various reports, our data indicate that the up-regulation of cFLIP may act as regulator of death ligand sensitivity also in fully mature DCs in the murine model system (Balkir et al. 2004; Golks et al. 2005; Irmler et al. 1997). However, and due to my experimental system I could not show the maturation dependent upregulation of CD95 surface receptor expression, which might additionally contribute to the detectable resistance of DCs to death ligands.

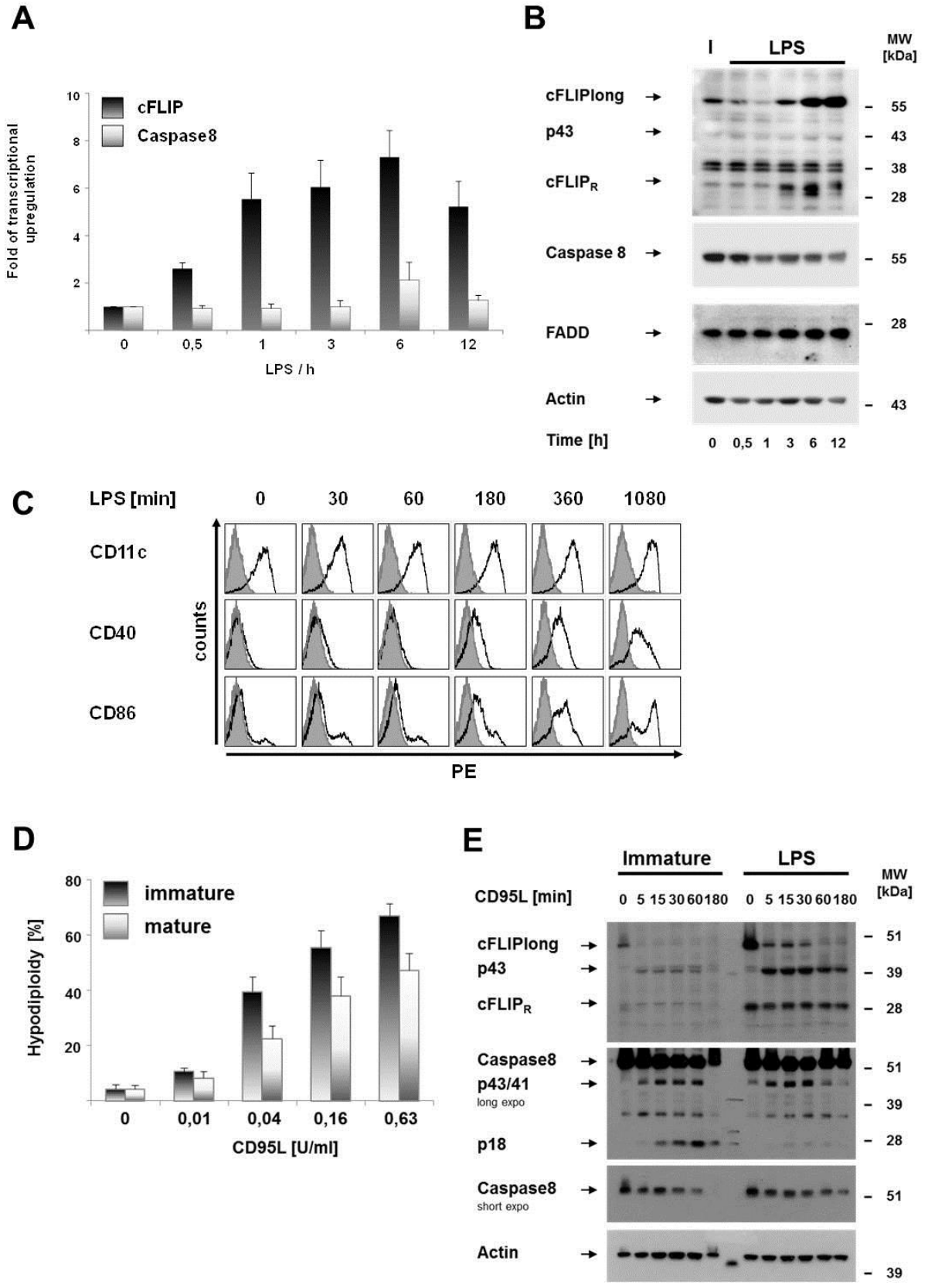

A
cFLIP
Caspase8
Fold of transcriptional upregulation
LPS / h
B
I
LPS
MW [kDa]
cFLIPlong
p43
cFLIPR
Caspase 8
FADD
Actin
Time [h]
C
LPS [min]
CD11c
CD40
CD86
counts
PE
D
immature
mature
Hypodiploidy [%]
CD95L [U/ml]
E
Immature
LPS
CD95L [min]
cFLIPlong
p43
cFLIPR
Caspase8
p43/41
long expo
p18
Caspase8
short expo
Actin

Figure 21

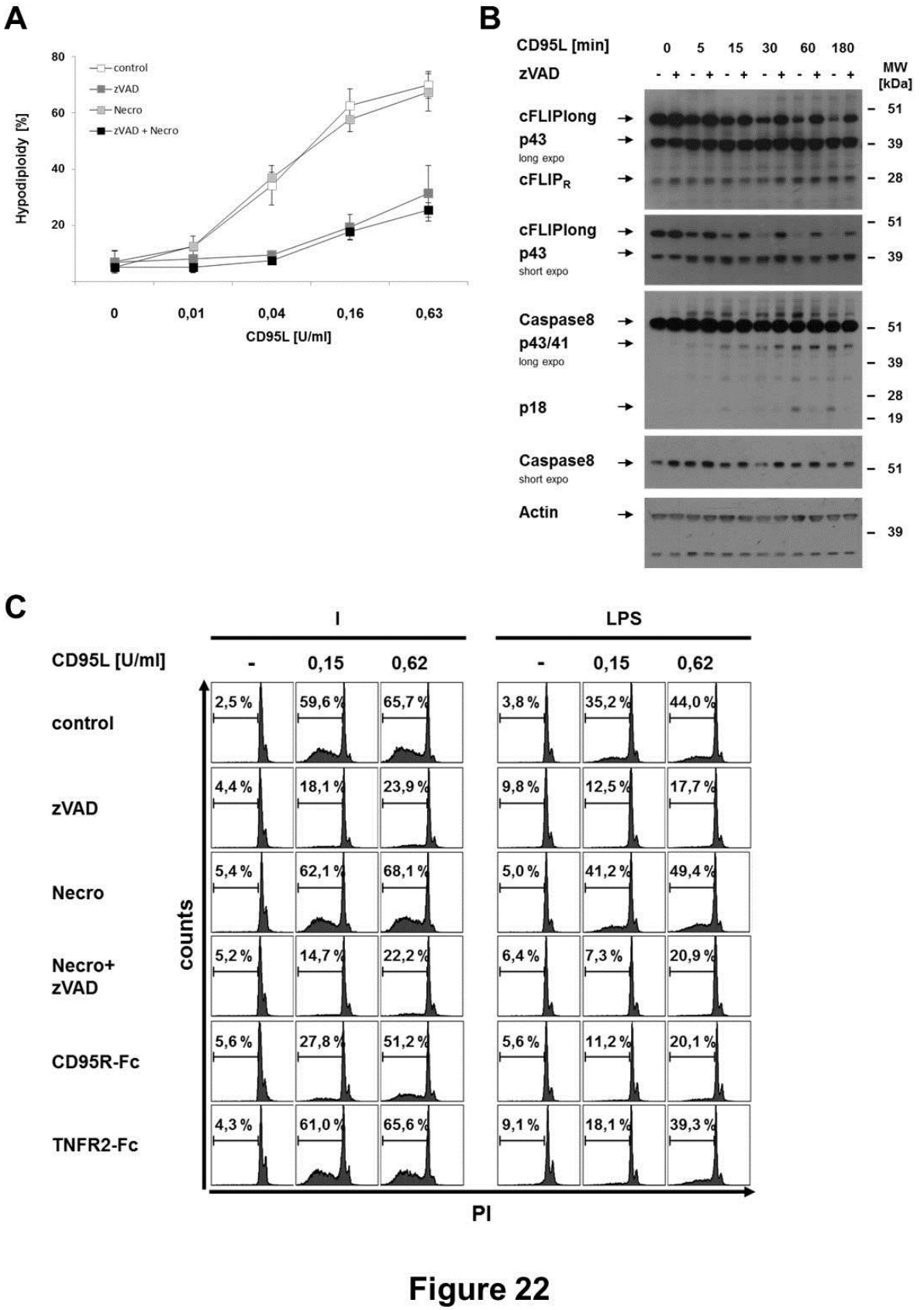
Figure 21: Expression of death receptor-associated signalling proteins in primary immature and LPS-matured DC and their sensitivity to CD95L-induced cell death.
Primary BMDC were matured for indicated time periods with LPS (1µg/ml). (A) mRNA expression analysis of cFLIP and caspase-8 in immature and mature DCs were performed by qPCR. The error bars represent SEM of three independently performed and summarized experiments. (B) Protein expression of cFLIP, caspase-8 and FADD of immature (I) and mature DCs after stimulation with LPS for indicated time points was analysed by Western blotting. Expression level of β-actin served as internal loading control. Data are representative for three independently performed experiments. (C) Analysis of cell surface expression of CD11c, CD40 and CD86 on matured and immatured DCs by FACS analysis. (D) Cell viability of DCs was analysed upon 6 hours in presence or absence of LPS and after 16 hours of CD95L stimulation by Nicoletti staining assay for indicated time. The error bars represent standard error of mean (SEM) of three independently performed and summarized experiments. (E) Expression of cFLIP and caspase-8 in immature and mature DCs was analysed by Western blot analysis after stimulation with CD95L for the indicated time points.

3.4.2. Characterisation of CD95L-induced cell death in BMDCs.

Apoptotic cell death is strongly regulated by active caspases while necrotic cell death is critically controlled by the kinase activity of RIPK1. To identify if the active kinase of RIP1 (RIPK1) also plays a role in CD95L-induced cell death in murine DCs and if the detected cell death is caspase dependent, experiments were performed with the pan-caspase inhibitor zVAD-fmk for inhibition of caspase processing in mature DCs and with the recently described RIPK1 inhibitor necrostatin-1 (Degterev et al. 2008) (Figure 22 A, C). Caspase-8 processing in mature DC was inhibited in the presence of the pan-caspase inhibitor zVAD-fmk (Figure 22 C); inhibition of RIPK1 activity had no influence on CD95L-induced cell death (Figure 22 A, C). Generated data show that stimulation of murine BMDCs with CD95L leads to an apoptotic cell death and not to a RIPK-1-dependent necrotic cell death. Within the mature DC population, I observed a delayed and inhibited processing of caspase-8 consistent with a decreased sensitivity to apoptotic cell death (data not shown). In contrast, caspase-8 cleavage in immature DC population was detected already after 15 minutes of stimulation with CD95L (Figure 22 B). These observed results indicate a rapid induction of the apoptotic signalling cascade in immature DCs upon presence of CD95L. Maturation induces an upregulation of anti-apoptotic molecules. In this case,

DCs life span determining the amplitude and the duration of immune responses gets prolonged. DC cell death is controlled by BCL-2–related proteins and death receptors (Lehner et al. 2012). Death receptor-mediated pathways do not act independently and are interconnected to a TRAIL-R as well as TNFR1-signalling network through various mechanisms. CD40 and receptors of the TNF receptor superfamily trigger pro- and anti-apoptotic pathways, and the activity of NF-κB determines whether signals from these receptors are perceived as survival or as death signals in DCs (Green and Llambi 2015; Kriehuber et al. 2005; Ludewig et al. 1995; McLellan et al. 2000; Wong et al. 1997). Lehner et al. could provide evidence that TNF stimulation (especially autocrine TNF) via TNF receptor 1 (TNFR1) can trigger both pro- and anti-apoptotic pathways in particular after activation of DCs by TLR agonists (Lehner et al. 2012). To exclude the possibility that autocrine TNF production was responsible for the sensitivity to CD95L, a soluble TNF-R2-Fc was used to block autocrine TNF signalling. Indeed, TNF-R2-Fc treatment was ineffective to protect from CD95L-induced cell death in immature DCs, but partially protective in mature DCs. These results argue for an additional effect of autocrine TNF produced during CD95L-induced death signalling in mature DCs (Figure 22 C). So, TNF (paracrine and autocrine) has an impact on the life span of professional antigen presenting cells and thus on the extent of the immune response. However, CD95R-Fc treatment protected immature and mature DCs from CD95L-induced cell death to the same extend as blocking of caspases by zVAD-fmk.

Summarizing these data, during DC maturation the resistance to death receptor-mediated apoptotic cell death results mainly from upregulation of cFLIP. The observed CD95L-induced cell death is caspase-dependent. In addition, our data confirm, that the CD95-mediated pathway in mature DCs is interconnected to the TNFR1-signalling network. Autocrine TNF signalling seems to play a protective role in mature DCs and I conclude that this process is required for a prolonged stimulation of TCs and allows for a more efficient amplitude and duration of immune responses.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Hypodiploidy [%]
control
zVAD
Necro
zVAD + Necro
CD95L [U/ml]
0
0,01
0,04
0,16
0,63
B
CD95L [min]
0 5 15 30 60 180
zVAD
MW [kDa]
cFLIPlong
p43
long expo
cFLIP$_R$
cFLIPlong
p43
short expo
Caspase8
p43/41
long expo
p18
Caspase8
short expo
Actin
C
I
LPS
CD95L [U/ml]
control
zVAD
Necro
Necro+ zVAD
CD95R-Fc
TNFR2-Fc
counts
PI

Figure 22

Figure 22: Characterisation of CD95L-induced cell death in BMDCs.
Primary BMDCs, either immature or matured by 6 hours LPS (1µg/ml) incubation, were treated with indicated concentrations of CD95L. (A) Cell viability of immature DCs pre-treated for 1 hour either separately or in combination with zVAD-fmk (20 µM) and Necrostatin-1 (50 µM) was analysed after CD95L stimulation for indicated concentrations by Nicoletti staining assay. The error bars represent standard error of mean (SEM) of three independently performed and summarized experiments. (B) Protein expression of cFLIP and caspase-8 in immature DCs was analysed by Western blot analysis after stimulation with CD95L for the indicated time points in the presence or absence of caspase inhibitor zVAD-fmk (20 nM). (C) Immature and mature DCs were either separately or in combination pre-treated with CD95-Fc (10 µg/ml), 1h), TNFR2-Fc (10 µg/ml, 1h), zVAD-fmk (20 µM; 1h), Necrostatin-1 (50 µM, 1h) and subsequently stimulated with the indicated concentration of CD95L. Viability of cells was analysed by Nicoletti staining assays as described before. The FACS plots shown are representative for three independently performed experiments.

3.4.3. DISC formation in primary DCs

Next, the death-inducing signalling complex (DISC) was examined for analysing, which molecules play a role in proximal CD95 signalling and for further characterisation of the CD95L-mediated apoptotic signalling pathway in the used DC model system. The receptor functionality in immature and LPS-matured BMDCs was tested by ligand affinity precipitation using human CD95L-Fc that cross-reacts with murine CD95 (Bossen et al. 2006) (Figure 24). Stimulation of CD95 receptor in DCs led to SDS- and β-mercaptoethanol-insoluble CD95 receptor complexes of higher molecular mass (Feig et al. 2007), their composition was analysed in Western blots of DISC precipitates. Upon precipitation of the CD95-DISC in immature as well as in mature BMDCs stimulated with CD95L, the recruitment of cFLIP, caspase-8 and FADD was detected (Figure 23). In line with previous reports, the cFLIP p43 cleavage fragment was mainly recruited to the DISC (Figure 23) (Diessenbacher et al. 2008; Scaffidi et al. 1999b). These results indicate a rapid DISC-associated caspase-8-mediated cleavage of cFLIP. As expected and due to the upregulation of cFLIP expression upon maturation, the cFLIP recruitment in LPS pre-treated BMDCs was enhanced than compared to immature DCs. FADD and caspase-8 recruitment were also detected in both DC populations. These data demonstrate for the first time DISC formation and composition in DC populations. The complex-formation in murine DCs is maturation-dependent and argues that cFLIP protects BMDC from death

receptor-mediated cell death at the level of the DISC by inhibition of caspase-8.

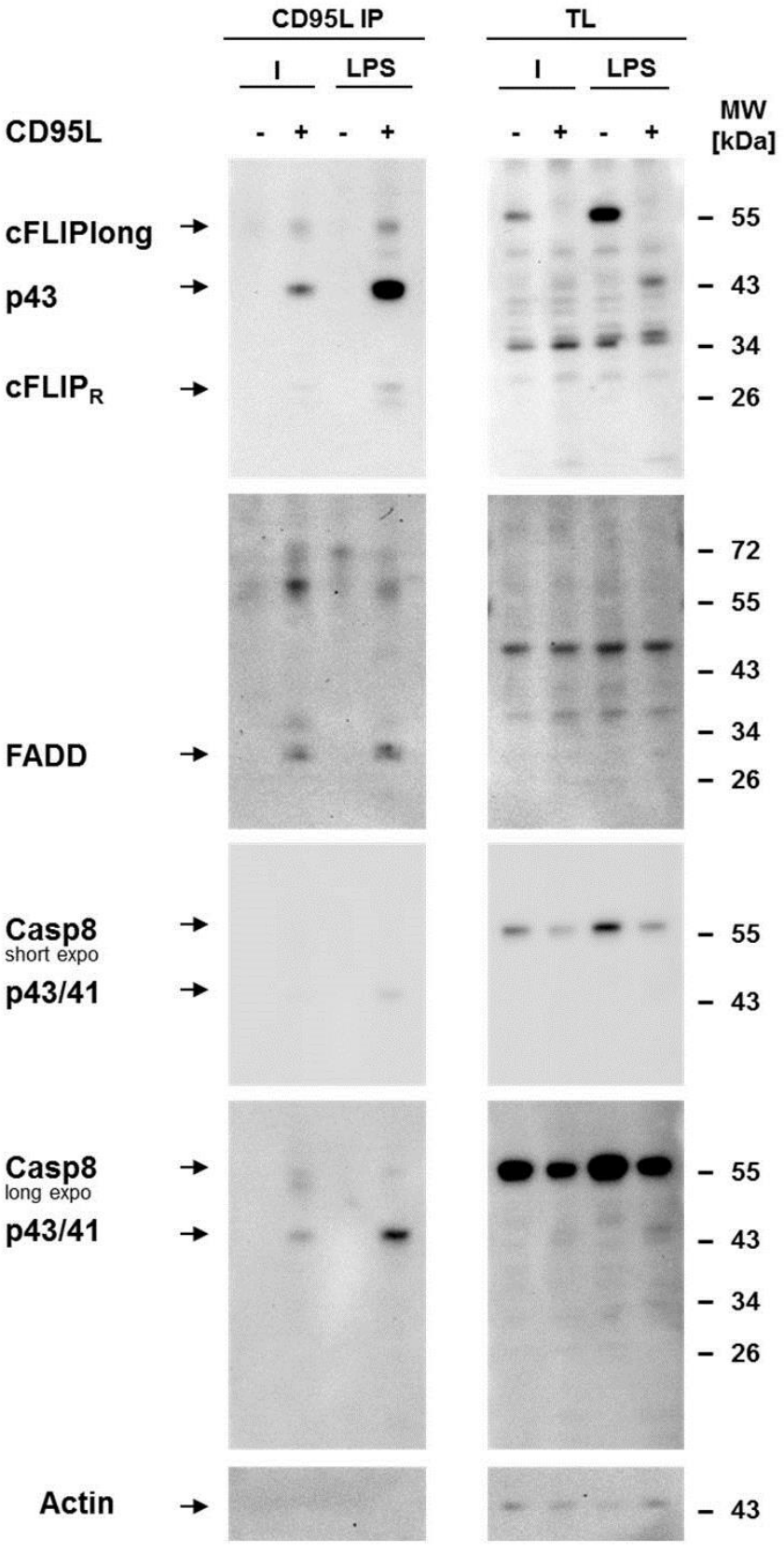


Figure 23

Figure 23: Immunoprecipitation of the CD95-DISC in immature and mature primary murine DCs.
Primary BMDCs, either immature (I) or matured by 6 hours LPS (1 µg/ml) incubation were treated for 30 minutes with 250 U/ml CD95L-Fc, formation of the ligand-induced receptor-bound CD95 complex (DISC) was induced. CD95L-Fc immunoprecipitation was performed as explained in detail in materials and methods. Precipitation of receptor complexes after lysis (-) served as internal specificity control when compared with ligand affinity precipitates (IP; +). Equal amounts of DISC (CD95L IP were subsequently analysed by Western blotting for the indicated molecules. For comparison of signal strength between CD95L-IP and total cellular lysates (TL), equal amounts of total cellular lysates were loaded on the same gels. Shown is one out of three representative experiments.

Summarizing these data, DCs sensitivity to CD95-induced apoptosis is strongly dose dependent and inversely correlates with their maturation state. Consistent with an apoptotic cell death, I observed processing of caspase-8 within 15 minutes in the immature, but not in mature DCs (Figure 21). Stimulation with CD95L induces recruitment of FADD, cFLIP and caspase-8 to the DISC within 30 minutes in immature and mature primary DCs. So finally, the cFLIP expression is maturation dependent and protects BMDCs from CD95L-mediated cell death by inhibition of caspase-8 at the DISC.

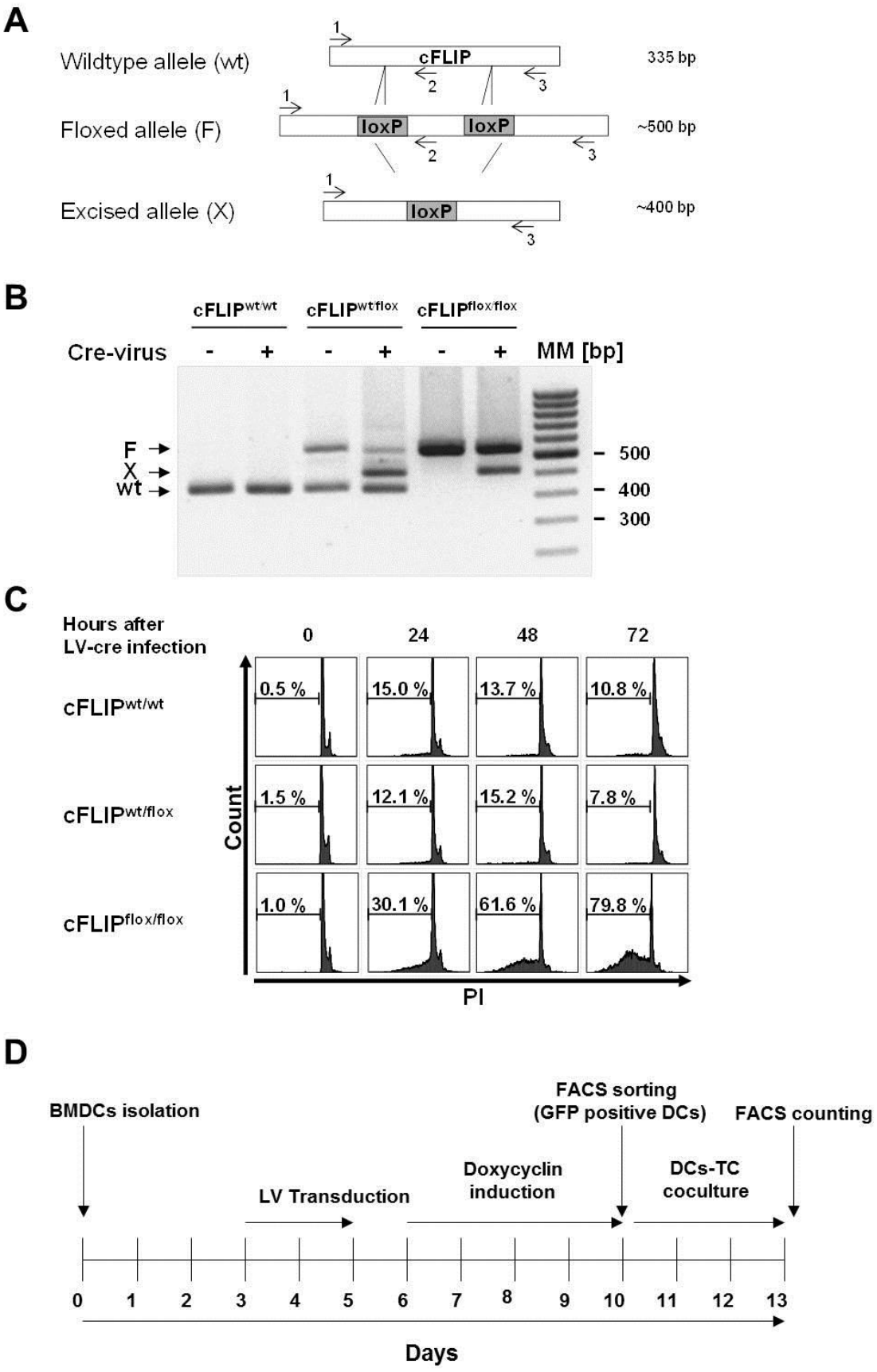
For further analysis concerning the role of cFLIP especially in CD95L-induced cell death in murine DCs, three strategies can be applied: 1. lentiviral over expression of cFLIP, 2. genetic deletion of the cFLIP locus and 3. cFLIP down regulation by specific siRNAs in murine BMDCs. All three strategies were based on the use of lentiviral expression systems with 2nd and 3rd generation packaging system. Due to the fact that overexpression of cFLIP reproduces the maturation dependent effects and may not reflect the physiological situation in detail this part of this work focused on the knock-out and knock-down approach.

3.5. Impact on DC lifespan upon a loss of cFLIP

3.5.1. Genetic deletion of the cFLIP locus results in spontaneous cell death

Based on the CD95L-induced apoptotic sensitivity of immature BMDCs, I hypothesized that a complete loss of cFLIP would lead to a spontaneous cell death. For the complete genetic deletion of the cFLIP locus, the Cre-mediated recombination technology was applied in order to excise cFLIP from primary DCs of a transgenic mouse model bearing the cFLIP gene flanked by loxP sites ($cFLIP^{fl/fl}$) (Yeh et al. 2000; Zhang and He 2005). Heterozygous animals bearing the loxP site only in one allele ($cFLIP^{wt/fl}$) or wild type animals ($cFLIP^{wt/wt}$) served as controls. Upon isolation of murine DCs from respective mice, cells were transduced with a lentiviral vector encoding Cre recombinase. The cFLIP excision in the tranduced DCs by Cre recombinase activity was confirmed by multiplex PCR 24 hours after viral infection (Panayotova-Dimitrova et al. 2013) (Figure 24 A and B). In homozygous animals, no wildtype allele was amplified (Figure 24 B, lane 5 and 6). Only upon Cre recombinase activity, the recombination-induced knockout of cFLIP (X) was detected additionally to the floxed allele (F) (Figure 24 B, lane 6).

Within a couple of hours after infection a rapid spontaneous cell death of transduced $cFLIP^{fl/fl}$ DCs was observed by microscopic examination, which was further confirmed by flow cytometric analyses of Nicoletti staining (Figure 24 C). In contrast, cells expressing wild type cFLIP at least in one allele ($cFLIP^{wt/fl}$ and $cFLIP^{wt/wt}$) were protected from spontaneous cell death. These findings confirm the hypothesis that complete absence of cFLIP leads to rapid spontaneous apoptosis and the presence of at least one allele of the cFLIP gene is sufficient to effectively protect from spontaneous cell death. Due to the fact that complete loss of cFLIP is accompanied by spontaneous cell death in primary DCs, an *in vitro* knockdown approach for elucidating the role of cFLIP in those cells should be established. An experimental scheme for the following knockdown experiments is shown in Figure 24 D.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Wildtype allele (wt)
cFLIP
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Floxed allele (F)
loxP
loxP
~500 bp
Excised allele (X)
loxP
~400 bp
1
2
3
B
cFLIPwt/wt
cFLIPwt/flox
cFLIPflox/flox
Cre-virus
- + - + - +
MM [bp]
F
X
wt
500
400
300
C
Hours after LV-cre infection
0
24
48
72
cFLIPwt/wt
0.5 %
15.0 %
13.7 %
10.8 %
cFLIPwt/flox
1.5 %
12.1 %
15.2 %
7.8 %
cFLIPflox/flox
1.0 %
30.1 %
61.6 %
79.8 %
Count
PI
D
BMDCs isolation
LV Transduction
Doxycyclin induction
FACS sorting (GFP positive DCs)
DCs-TC coculture
FACS counting
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days

Figure 24

Figure 24: cFLIP protects from spontaneous cell death in murine DCs.
BMDC isolated from cFLIP$^{fl/fl}$, cFLIP$^{+/fl}$, and cFLIP$^{+/+}$ mice (Zhang and He 2005) were transduced with Cre recombinase encoding lentivirus (Cre-LV). (A) Schematic representation of the design of the multiplex PCR for detection of the deleted cFLIP allele. (B) Detection of the Cre-mediated excision of the cFLIP allele (x) in DCs. DNA was isolated from all samples and analysed by multiplex PCR for the floxed allele (F), wild type allele (wt), or the excised allele (X). (C) Cell viability of immature and mature DCs was determined by Nicoletti staining assay, at the indicated time points after Cre-LV transduction. The FACS plots shown are representative for three independently performed experiments. (D) Schematic illustration of the design of the LV-transduction experiments.

3.5.2. Knockdown of cFLIP increases CD95L-induced cell death in primary BMDCs

Using RNA interference for a partial silencing of cFLIP (cFLIP knockdown) would most likely allow circumvention of the limitation of a complete genetic kockout system. In order to maximise the control of cFLIP silencing in primary DCs, a lentiviral vector based on tTRKRAB-mediated gene control system (Szulc et al. 2006) allowing for a tetracycline-controllable RNA interference of cFLIP (LV-cFLIP-siRNA) was generated. Efficiently transduced primary DCs were cultured in the presence of tetracycline in order to induce siRNA expression. Successfully transduced cells were identified by their GFP expression and were subsequently sorted by FACS for further functional and biochemical experiments (Figure 25). The repression of cFLIP expression in LV-cFLIP-siRNA–transduced BMDCs as compared to the LV-control–transduced BMDCs was confirmed by both real-time quantitative RT-PCR and Western blotting (Figure 25 A, B). When compared to wildtype (wt) and control vector (cv) transduced DCs, cFLIP expression is approximately 50 % downregulated on mRNA level (Figure 25 A). Additionally, the cFLIP$_{long}$ protein, caspase-8 as well as actin expression is diminished in the LV-cFLIP-siRNA-transduced BMDCs compared to control vector transduced cells (Figure 25 B). I next evaluated the impact of cFLIP down regulation on CD95L-mediated apoptosis in primary DCs. PI staining of hypodiploid apoptotic nuclei as well as fluorescence microscopy studies were performed to characterize the observed cell death and its morphology (Figure 25 C-F). Cells were stained with Hoechst-33342, to detect the chromatin condensation during apoptosis and with Sytox Green (high-affinity nucleic acid stain), which is able

to stain nucleus only when the integrity of the membrane is disrupted allowing the detection of late apoptotic events and also necrosis. Increased numbers of typical apoptotic cells demonstrating membrane blebbing, DNA condensation and fragmentation were detectable in the cFLIP-knockdown BMDCs (Figure 25 D). PI staining of infected BMDCs during 72 hours doxycycline-induction of LV-gene expression already revealed increased cell death in LV-cFLIP-siRNA transduced primary DCs when compared with control transduced cells (Figure 25 E, F). Our data thus indicate that suppression of cFLIP expression directly impacts on the lifespan of DCs. The expression of cFLIP is indispensable for DC cell survival and plays probably an important role during DC-TC interaction limiting and determining the immune response and TC differentiation.

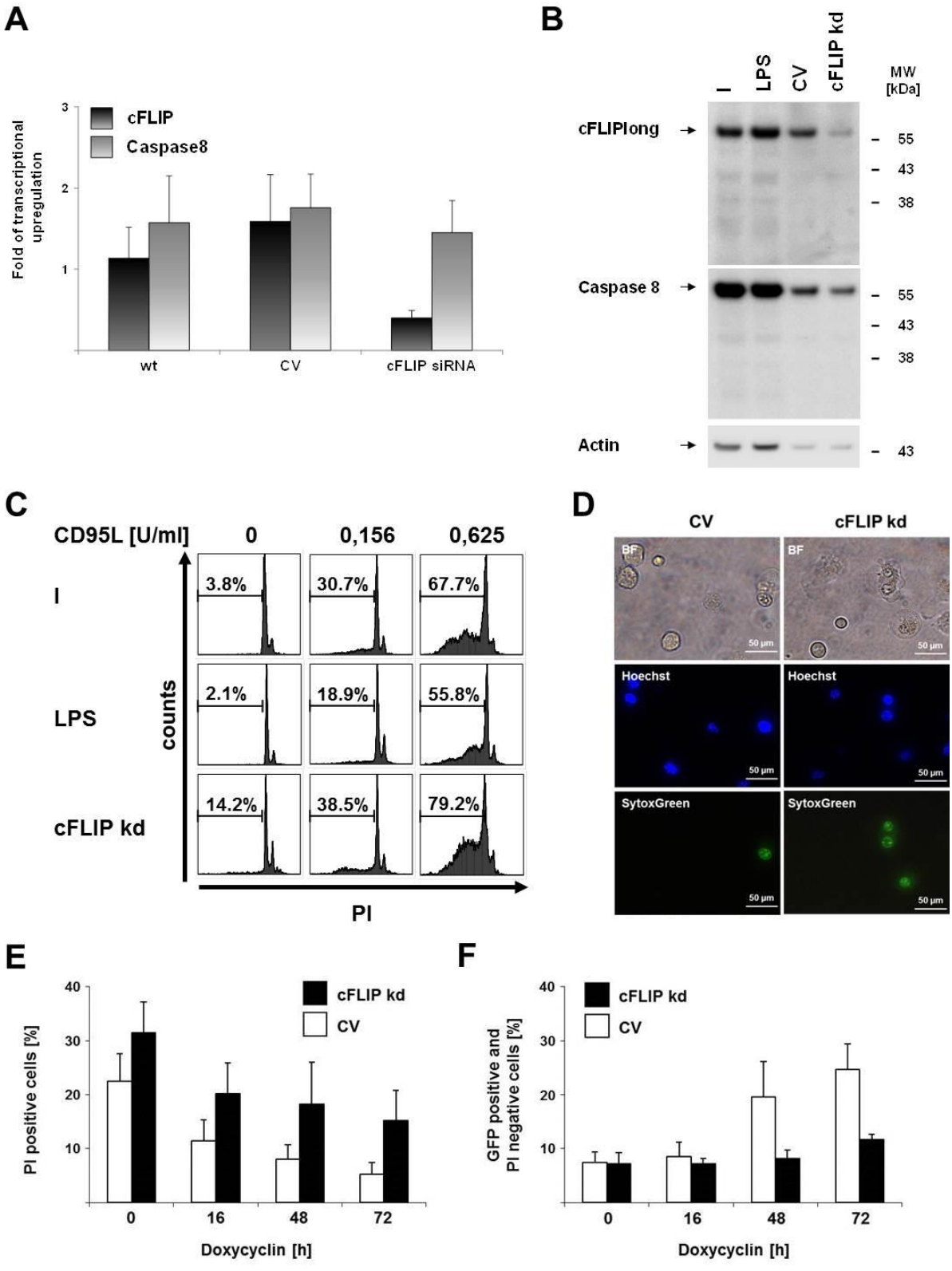
A
Fold of transcriptional upregulation
cFLIP
Caspase8
wt
CV
cFLIP siRNA
B
I
LPS
CV
cFLIP kd
MW [kDa]
cFLIPlong
Caspase 8
Actin
55
43
38
C
CD95L [U/ml]
0
0,156
0,625
I
LPS
cFLIP kd
3.8%
30.7%
67.7%
2.1%
18.9%
55.8%
14.2%
38.5%
79.2%
counts
PI
D
CV
cFLIP kd
BF
Hoechst
SytoxGreen
50 µm
E
PI positive cells [%]
cFLIP kd
CV
Doxycyclin [h]
F
GFP positive and PI negative cells [%]
cFLIP kd
CV
Doxycyclin [h]

Figure 25

Figure 25: Inducible knockdown of cFLIP in primary murine DCs.
Primary DCs were transduced with cFLIP-siRNA-LV (cFLIP kd) or Control-siRNA-LV (CV), respectively. After tetracycline induction for 4 days, GFP-positive cells were FACS sorted and analysed for cFLIP expression.
(A) mRNA expression of cFLIP and caspase-8 in mock-infected, cFLIP-siRNA-LV or Control-siRNA-LV BMDCs were analysed by qPCR. The error bars represent SEM of three independently performed experiments. (B) Protein expression of cFLIP and caspase-8 upon transduction with cFLIP-siRNA-LV or Control-siRNA-LV was analysed by Western blotting, expression level of β-actin served as internal loading control. (C) Cell viability of immature (I), mature (LPS) and cFLIP-siRNA-LV (cFLIP kd) DCs was analysed by Nicoletti staining assay, 16 hours after addition of the indicated concentrations of CD95L. The FACS plots shown are representative for three independently performed experiments. (D) For characterisation of cell death, BMDC morphology was investigated by simultaneous staining with Hoechst-33342 (5 µg/ml) and Sytox Green (5 pM) for 15 min at 37°C, immediately followed by transmission and fluorescence microscopy, respectively. One representative of a total of three independent experiments is shown. (E, F) After 0, 16, 48, 72 hours tetracycline induction of LV-expression, GFP expression and viability of BMDCs by PI staining was determined by FACS analysis. The error bars represent standard error of mean (SEM) of three independently performed and summarized experiments.

3.5.3. Impact of cFLIP downregulation in DCs on T cell activation

Preliminary investigations in murine and human mature DCs established high resistance to apoptosis adverse exogenously added CD95L (Leverkus et al. 2000b; Leverkus et al. 2003a). However, it cannot be excluded that during cell-cell interactions the lack of TC surviving signals (CD40L, TRANCE and other until now unknown factors) *in vivo* modulate the apical CD95 signalling cascade and so sensitizes DC for apoptotic signals. I now hypothesized that a diminished cFLIP expression in our DC model system could probably lead to the reduction of TC proliferation due to the augmented CD95L-sensitivity of BMDCs and the reduced DC life span during interaction. Upon characterisation of the CD95 signalling cascade in murine DC also in context of modulated cFLIP expression, antigen-specific DC/TC interaction analysis were performed to determine DC capacity to activate TCs. *In vitro* interaction assays with DCs bearing a cFLIP knockdown co-cultured with TCs were performed. Similar to functional analysis outlined in Figure 24 D, primary DCs were infected and subsequently FACS-sorted. An immunological synapse with chicken $Ovalbumin_{323-339}$-specific $CD4^+$ TCs isolated from the well-established OT2 mouse

model was simulated *in vitro*. Under our experimental conditions, I was unable to confirm the hypothesis and to detect significant changes in the capacity of TC activation compared to control cells as a result of cFLIP knockdown (Figure 26). However, I noted that LV-transduction of DCs alone leads to a reduction of TC proliferation rate (Figure 26 B, C). Furthermore, I observed no significant changes in the viability of DCs or TCs during the *in vitro* co-culture (Figure 26 D, E). Independent on the DC infection, the amount of cells dying within the interaction model system was below 2 %. The application of the experimental system well-working in model cell lines is limited in primary DCs due to the long experimental tetracycline-induction of siRNA expression (Figure 24 D).

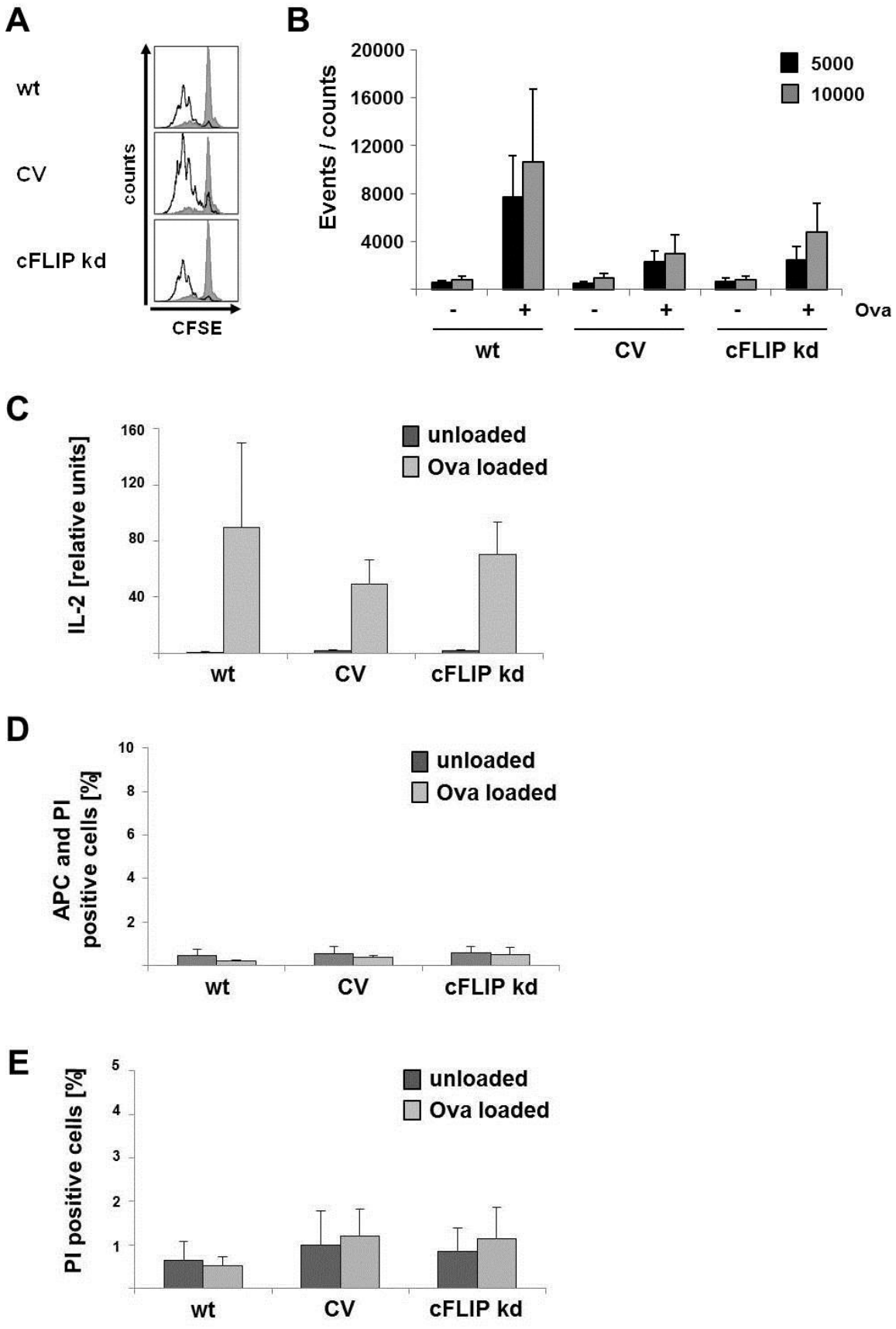
A
wt
CV
cFLIP kd
counts
CFSE
B
Events / counts
20000
16000
12000
8000
4000
5000
10000
-
+
-
+
-
+
Ova
wt
CV
cFLIP kd
C
IL-2 [relative units]
160
120
80
40
unloaded
Ova loaded
wt
CV
cFLIP kd
D
APC and PI positive cells [%]
10
8
6
4
2
unloaded
Ova loaded
wt
CV
cFLIP kd
E
PI positive cells [%]
5
4
3
2
1
unloaded
Ova loaded
wt
CV
cFLIP kd

Figure 26

Figure 26: cFLIP expression level of BMDCs does not modulate the quantity of *in vitro* stimulation of T cells
Primary BMDCs were either not infected (wildtype – wt) or transduced with cFLIP-siRNA-LV (cFLIP kd) or Control-siRNA-LV (CV), respectively. After tetracycline induction for four days, GFP-positive cells were FACS sorted and co-cultivated with $CD4^+$ OVA-specific T cells. (A and B) Cell proliferation of CFSE labelled T cells was measured after 60 hours of co-cultivation. (C) The secretion of the cytokine IL-2 was determined by ELISA. Data are representative of five independently performed experiments and are expressed as mean values ± SE (D and E). Cell viability after 60 hours co-culture was determined by PI and APC staining by FACS analysis. Summarized data are representative of five independent experiments and are expressed as mean values ± SE.

Summarizing the results, lentivirus infected as well as uninfected DCs induce TC proliferation in the used experimental model system. Downregulation of cFLIP in DCs had no impact on TC activation. Interestingly the TC proliferation rate is reduced if DCs were infected with lentivirus independent of cFLIP knockdown, which needs further investigation. No significant amount of dead cells during interaction could be detected regardless whether cFLIP is downregulated in DCs or not. So, downregulation of cFLIP had no impact on cell survival especially for DCs within the interaction model system. However, the long experimental procedure may be one aspect for the generated results. Beside the diminished TC proliferation rate upon co-culture with lentiviral infected DCs the direction of immune response still remains open and needs further analysis.

4. Discussion

4.1. Insights into inflammatory signalling pathways in DCs with respect to IL-1β

Professional antigen presenting cells such as DCs are indispensable for an organism because they play a key role for initiation and activation of the immune response as well as tolerance (Janeway, Jr. et al. 2012). They represent an attractive potential therapeutic target as vehicles for immune-suppressive therapeutic factors due to their migratory capability and the essential role in maintaining tolerance (Toscano et al. 2010). Investigation of the role of inflammasomes for maturation and antigen presentation as well as the inflammasome function and the CD95L signalling in DCs would allow for a more complete molecular understanding of the underlying cell biology and of the molecular interactions involved in immune responses. Based on cell morphology, expression profile of specific markers and functional attributes, DCs have been defined. However the heterogenous family of cells is capable to suppress and activate immune responses dependently and independently of T cell activation or migration to secondary lymphoid organs (Arora et al. 2014; Mashayekhi et al. 2011; Ochando et al. 2006; Reis e Sousa 2006; Satpathy et al. 2013; Whitney et al. 2014). Due to a common bipotent progenitor and overlapping functions, an unambiguous distinction of DCs from monocytes and macrophages by phenotypic and functional analysis still remain difficult (Schraml and Reis e Sousa 2015). For a distinction of DCs from monocytes and macrophages CD11c and CD68 surface expression analysis provided the technical base in this work.

DC differentiation, DC maturation, inflammatory processes and immune responses depend on cellular background and environment, especially on cytokines that activate, trigger or repress signalling pathways (Greter et al. 2012a; Greter et al. 2012b; Watanabe et al. 2008). Furthermore, antigen uptake and processing, triggering of TLRs and NLRs by ligands like PAMPs and DAMPs or the interaction with other molecules like CD40 and CD40L activate DCs and induce a line of phenotypical, morphological and functional changes in immature DCs (Jonuleit et al.

1997; Manickasingham and Reis e Sousa 2000; Michelsen et al. 2001; Reddy et al. 1997; Sallusto et al. 1995).

DCs transition from an antigen incorporating cell to an antigen presenting cell was for example characterized by the loss of the ability to uptake and process an antigen here shown for dextran (Fig. 7 B). Simultaneously this DC maturation was accompanied by the increased expression of different costimulatory and adhesion molecules on the cell surface like CD80, CD86 and CD40, which are essential for TC activation at the immunological synapse as well as MHC class II molecules (Fig. 7 A) (Banchereau and Steinman 1998; Bell et al. 1998; Ganpule et al. 1997; Hauss et al. 1995; Inaba et al. 1989; Inaba et al. 1993; Prickett et al. 1992; Xu et al. 1992). In my study, I showed that beside the commonly used LPS (Janeway, Jr. et al. 2012) a defined cocktail of several components, namely TNF-α, IL-4, CD40L and PGE2 was another stimulus for producing mature mouse bone marrow-derived DCs, which was shown for human DCs (Jonuleit et al. 1997; Leverkus et al. 2000b) and that I can reliably monitor DC maturation in my cellular model system.

4.1.1. Cytokine production upon pathogen recognition

Beside DC maturation, the recognition of microbial, viral or extracellular substances termed PAMPs and DAMPs by DCs lead to formation and activation of the inflammasome, which processes pro-forms of inflammatory cytokines like IL-1β and IL-18 (Freche et al. 2007). This leads to the activation of these cytokines and under certain circumstances to their subsequent release.

First, I characterized the impact of maturation by either LPS or the cytokine cocktail on some molecules of the inflammatory signalling pathway and found an upregulation of the inflammatory cytokines IL-1β, IL-18 and IL-33 on the mRNA level, and of pro-IL-1β and Caspase-11 on the protein level (Fig. 7 C, D) as well as a subsequent IL-1β release (Fig. 8 C) upon DC maturation. To my knowledge, I demonstrated for the first time that murine DCs can be matured with a cocktail using the CD40 agonism in conjugation with TNF-α, IL-4 and PGE-2 like shown for human DCs (Leverkus et al. 2000b), and that this maturation process leads by a yet to be defined mechanism to an inflammasome activation and subsequent IL-1β secretion. Multiple different

signalling cascades activated by the cocktail-induced DC maturation probably make the interpretation of generated data more difficult and complex (Decker and Shpall 2008). To improve conclusions about the correlation between cytokine expression, subsequent secretion and DC maturation, I mainly focussed on one maturation substance like LPS. I additionally compared different categories of maturation stimuli that were available in clinical grade for their ability to upregulate pro-IL-1β expression and to induce IL-1β secretion.

From the TLR4 stimuli Lipopolysaccharide (LPS) and Heparan Sulfate (HepS) and the Nod2 ligand muramyldipeptide (MDP), all known to induce for example the production of pro-inflammatory cytokines (An et al. 2002; Kaisho et al. 2001; Kodaira et al. 2000; Medzhitov and Janeway, Jr. 1998; Poltorak et al. 1998a; Poltorak et al. 1998b; Spirig et al. 2008; Takeuchi and Akira 2010; Wright et al. 1990) only LPS stimulation of DCs resulted in upregulation of pro-IL-1β on the protein level and in secretion of the cytokine within the time tested (18 hours) (Kaisho and Akira 2001; Poltorak et al. 1998b; Tsan and Gao 2004). Although cleavage and shedding of heparan sulfate, proteoglycans and glycosaminoglycan fragments was observed rapidly after injury of tissues (Ihrcke and Platt 1996; Kainulainen et al. 1998), I could not detect IL-1β secretion by DCs after 18 hours stimulation in the used cellular model system consistent with generated data by Kodaira, Nair et al. in 2000, where earliest IL-1β secretion was detected only after 24 hours stimulation (Kodaira et al. 2000). Confirmed by my analysis, MDP, which is a minimal structural unit of peptidoglycan within the bacterial cell wall skeleton, exhibited no ability to mature DCs and to induce IL-1β release by DCs (Tsuji et al. 2000; Uehori et al. 2005).

However, stimulation of TLR2 with peptidoglycan (PGN), hyaluronic acid (HA) or lipoteichonic acid (LA) (gram pos bact cell wall components) led to an upregulation and secretion of IL-1β (Figure 7E) consistent with Schwandner et al. (Kaisho and Akira 2002; Schwandner et al. 1999; Takeuchi et al. 1999). All other tested DC maturation stimuli did not lead to IL-1β secretion within the used time period of 18 hours (Alexopoulou et al. 2001; Apostolopoulos and McKenzie 2001; Berchtold et al. 1999; Cambi et al. 2008; la et al. 2002; Mutini et al. 1999; Sistigu et al. 2014).

In summary, LPS was the strongest inducer of the IL-1β secretion in my analysis. These findings indicate a main induction of the Il-1β signalling pathway via TLR4. Some specific substances like LA and HA induce the IL-1β signalling pathway via TLR2. The TLR3 receptor signalling pathway, which needs to be activated intracellularly in endosomes (Rajan et al. 2011), and the mannose receptor signalling pathways probably are not directly involved in the IL-1β cytokine release.

Since maturation of DCs induced by the cocktail containing TNF-α, IL-4, CD40L and PGE2 somehow additionally led to a strong activation of the inflammasome, characterisation of intracellular components of the inflammatory signalling cascade would allow for finding differences or similarities between LPS and cocktail-induced inflammasome activation. Monitoring DC maturation and IL-1β secretion after LPS or cocktail stimulation over time depicted a time dependency of both signalling cascades (Figure 8).

Both maturation stimuli induced upregulation of CD40, CD86 and pro-IL-1β (Aiba 1998; Lee et al. 2002; Lipscomb and Masten 2002; Reis e Sousa 2006; Schmidt et al. 2012; Winzler et al. 1997). LPS stimulation led to an upregulation of only Caspase-11. The upregulation of Caspase-1 upon LPS maturation as shown by Martinon et al. could not be confirmed / reproduced with the used antibody for detection even in PMA primed THP-1 cells (data not shown) (Martinon et al. 2002). Due to the fact that pro-IL-1β was upregulated and secreted upon LPS or cocktail-induced maturation, the cytokine seemed to play a substantial role in both differently induced immune responses. Comparison of LPS and cocktail induced DC maturation showed a diminished activation of Caspase-11 as well as a diminished IL-1β upregulation and secretion in cocktail stimulated DCs arguing for a diminished inflammasome activation using this maturation signalling cascade. So, activation of inflammasome-mediated signalling pathways is time dependent and differs according to the maturation stimulus in DCs.

Interestingly, upon maturation the genetic background of DCs determined the amount of secreted cytokines (IL-1β and IL-18) and already differed between animals of the same mouse strain C57/BL6J (Figure 9 B) probably due to epigenetic factors. According to these results, an interpretation of data needs to be done very carefully and led us to perform experiments with pooled DCs from different donors of the same mouse strain.

4.1.2. The trigger of the cytokine release

Numerous studies showed a release of endogenous ATP after LPS stimulation in monocytes (Ferrari et al. 1997; Ferrari et al. 2006; Petrilli et al. 2007) and that the subsequent uric acid- or ATP-stimulation leads to the induction of IL-1β and IL-18 secretion in an autocrine way in macrophages (MacKenzie et al. 2001; Piccini et al. 2008). For DCs, same biological functions mediated by these DAMPs have been suggested. Especially for ATP, the $P2X_7$ receptor seems to play a key role for the observed cytokine secretion in monocytes and macrophages (Franchi et al. 2007; Pelegrin et al. 2008). Indeed, my data showed that a stimulation of matured DCs with ATP or specifically active ATP-derivatives mainly trigger the $P2X_7$ receptor signalling pathway thereupon leading to IL-1β release (Figure 9 A, C). Whereas bz-ATP as specific activator of the $P2X_7$ receptor enhanced IL-1β release, ox-ATP partially blocked the IL-1β secretion. The oxidized ATP derivate has a weak affinity for the $P2X_7$ receptor, slow kinetics and many other pharmacological actions, which could be an explanation for the generated results that at best partially inhibited the signalling cascade (Burnstock 2007; Donnelly-Roberts et al. 2008; Surprenant 1996). In summary, my analysis nicely showed that ATP is one inducer of the observed IL-1β release depending on purinergic receptors by mature DCs mainly activating the $P2X_7$ receptor as demonstrated for monocytes and macrophages. Interestingly, upon DC-maturation and subsequent ATP stimulation, the secreted IL-1β molecule was detected only in unfiltered supernatants. Filtering with a 0.22 µm filter did not allow for the detection of the cytokine (data not shown) indirect supporting the hypothesis of a cytokine release in vesicles (Dinarello and Margolis 1995; Dubyak 2012; Pizzirani et al. 2007).

4.1.3. Differences between DCs and macrophages

As shown for ATP, I now suggest that stimulation of mature DCs with uric acid leads to inflammasome oligomerization as described for macrophages (Chen et al. 2006a; Liu-Bryan and Liote 2005; Martinon et al. 2006; Qu et al. 2007; Riteau et al. 2012; Scott et al. 2006). Therefore, I generated DCs and macrophages in parallel of the same pooled bone marrow cells and compared their surface expression profile, the

inflammatory protein expression profile as well as the IL-1β release after stimulation with ATP or MSU upon LPS maturation (Fig. 10). Due to the well-known cytotoxic effect of high levels of ATP over time (Aswad et al. 2005; Aswad and Dennert 2006; Coutinho-Silva et al. 1999; Peng et al. 1999) and for comparison of both DAMPs, the DC and macrophage exposure to ATP as well as MSU was not longer than 30 minutes in my experimental design. Interestingly, no significant effects on IL-1β secretion could be seen upon treatment with MSU neither for DCs nor for macrophages and ATP stimulation solely led to an IL-1β release in DCs. My data argue for a more crucial role of ATP and a more rapid signalling regarding the inflammasome activation in DCs. In macrophages, activation of the signalling cascade needed longer incubation times with much higher concentration of MSU (125-500 µg/ml instead of 5 µg/ml) and other crystals, namely 6 hours for macrophages were needed to see effects on IL-1β secretion, triggered by the extracellular delivery of endogenous ATP and dependent on purinergic receptors and connexin/pannexin channels (Riteau et al. 2012).

Additionally, the analysis and comparison of the expression profile for some inflammatory proteins revealed more differences between macrophages and DCs. I observed different expression levels for ASC and same levels for Caspase-1 (Englezou et al. 2015; Tan et al. 1997; Zanoni et al. 2016) and in contrast to Zanoni et al. different expression levels for Caspase-11 and pro-IL-1β. The expression level for ASC, Caspase-11 and pro-IL-1β were much higher in DCs than in macrophages arguing for a stronger activation of this signalling cascade in DCs. Caspase-11 expression augments in both cell types after LPS maturation. Furthermore, beside demonstration of cell-type specific differences in the inflammasome composition, inflammasome activation, $P2X_7$ receptor composition and cytokine regulation (Englezou et al. 2015; Hirsch et al. 2017) my data argue that in contrast to macrophages the IL-1β cytokine release as well as the inflammasome activation in LPS-matured DCs was much faster and mainly triggered by ATP activation of purinergic $P2X_7$ receptors. Uric acid does not seem to play an important role in the analysed inflammatory signalling pathway in DCs within the first 30 minutes of stimulation. Moreover, the lower protein expression level of pro-IL-1β, ASC and Caspase-11 independent on the maturation status of macrophages led me to the

hypothesis that the IL-1β release upon LPS maturation is much stronger and also faster activated in DCs than in macrophages.

In 2006, Franchi et al. showed that the activation of purinergic $P2X_7$ receptors by extracellular ATP is followed by a K+ efflux and activation of phospholipase A2, which is the key physiological inducer of a rapid cytokine release in macrophages (Franchi et al. 2006; Pelegrin et al. 2008). Furthermore, previous reports demonstrated a differential requirement for intracellular K+ in caspase-1 activation (Andrei et al. 2004; Fernandes-Alnemri et al. 2007; Franchi et al. 2007; Kahlenberg and Dubyak 2004a; Kahlenberg and Dubyak 2004b; Petrilli et al. 2007). Indeed, inhibiting the K+ efflux or caspase activity by zVAD-fmk led to an almost complete loss of IL-1β secretion in LPS-matured DCs and inhibition of caspase activity to an enrichment of pro-IL-1β and pro-Caspase-11 on protein level (Figure 11 A, B). So, the efflux of intracellular K+ mediated by the $P2X_7$ receptor as well as the activity of caspases is critical for the secretion of IL-1β in LPS-matured DCs.

Furthermore, a calcium-channel dependency was described for macrophages if potassium efflux was blocked and calcium influx was induced by ionomycin (Gallin 1984; Rosati et al. 1986). My hypothesis that the $P2X_7$ receptor is also involved in the increase of intracellular Ca^{2+}, membrane depolarization and the secretion of inflammatory cytokines by murine DCs in response to ATP could not be confirmed in my experimental system (Figure 11 C, D) again demonstrating the differences between macrophages and DCs.

For more detailed analysis of the intracellular IL-1β signalling cascade, I next inhibited the translation with CHX (Al-Sadi et al. 2008; Gentle et al. 2012). Within the tested time period, I could only observe a diminished pro-IL1β protein expression level in cell lysates (Figure 12). The IL-1β secretion level after ATP stimulation was not yet affected by inhibition of the translation arguing for a fast IL-1β processing upon maturation and storage of huge amounts of the cytokine within the mature DC. Furthermore, I conclude, that the secretion of inflammatory cytokines is mainly regulated at the $P2X_7$ receptor level and that the refill of microvesicles with cytokines by gene transcription and translation needs more time.

4.1.4. Inhibition of the inflammasome

Inflammasomes are critical information hubs of the innate immune system and cellular and microbial inhibitors are used by some bacteria and viruses as one way to overcome the immune system (Table1, introduction (Johnston et al. 2007)). For a more detailed analysis of the inflammasome in DCs and to complete the understanding of the underlying cell biology and of the molecular interactions involved, I established a system to interfere with the inflammasome under physiological conditions. Inhibition of inflammasome assembly by targeting the adaptor molecule ASC, one key molecule between the NLR and the caspase, besides blocking the caspase recruitment to the inflammatory complex, caspase autocleavage or caspase activation by targeting the Caspase-1 are some possibilities for pathogens to block the downstream signalling cascade in APCs, including the cytokine release diminishing the attraction of other immune cells and subsequent immune responses.

I suggest that blocking the inflammasome by expression of a POP-like dnASC would lead to complete interruption of the inflammasome function and subsequent cytokine secretion in DCs and allow for a better characterisation of inflammatory signalling pathways in DCs. In 2002, the over-expression of a dominant-negative version of the adaptor molecule ASC was successfully used by Martinon and Tschopp for the inhibition of the NALP3 inflammasome in THP-1 cells (Martinon et al. 2002).

In my thesis, I adopted dnASC that binds to and thereby blocks the PYD-domain of the adaptor molecule ASC and subcloned the protein into a lentiviral system. Lentiviral systems are suitable for a sufficient infection of many cell types, including post mitotic cells. However being known as natural sensors also for viruses (Breckpot et al. 2010), DCs can be infected with lentiviruses. This virus infection is somehow limited and additionally leads to DC maturation, which is confirmed by my data (Figure 13) (Liechtenstein et al. 2013; Nasr et al. 2014; Schmidt et al. 2012; Toscano et al. 2010; Xiao et al. 2012; Zhang et al. 2014).

Unfortunately, I was unable to reproduce the data of Martinon and Tschopp for dnASC, neither in THP-1 and DC2.4 cells (Figure 14) nor in XS52 cells (data not shown) (Martinon et al. 2002). So finally, I tested the hypothesis that an over-

expression of dnASC partially inhibits or completely blocks the IL-1β secretion of DCs by blocking caspase-1 function at the inflammasome in primary DCs.

At first, I was trying to infect DCs with two lentiviruses encoding dnASC-expression upon puromycin and hygromycin selection and 4-HT induction. By this 4-HT induction of gene expression, I hypothesize to minimize known dnASC self-oligomerization-mediated cytotoxic effects (Hasegawa et al. 2007; Masumoto et al. 2001; Richards et al. 2001). Interestingly, most of the cells were dying after selection even with a high amount of virus independent of the gene, arguing for an insufficient double infection of primary DCs (Figure 15). Even by multiple repetition of this double infection approach, I was unable to receive living infected primary DCs. I conclude that this system has limited applicability for antigen presenting immune cells.

To circumvent the double infection of DCs an “all in one” system was developed allowing simultaneous selection and gene induction. Testing this model system in primary keratinocytes, my data confirmed the hypothesis of an inflammasome-dependent IL-1β release upon UVB light exposure (Figure 16) previously shown by an ASC-siRNA mediated approach (Feldmeyer et al. 2007; Marionnet et al. 1997; Park et al. 2006; Skiba et al. 2005; Wiswedel et al. 2007). Thereupon, the dnASC-expression and function was analysed in primary DCs (Figure 17). The detected amount of dnASC compared to full length ASC was relatively low, arguing either for a low expression level of the molecule based on a low infection efficiency or an inefficient detection by the antibody that was developed against full-length protein. Additionally, the presented data in Figure 17 argued for a mixed cell culture upon viral infection including non-transgene expressing as well as dead cells during functional experiments. A diminished transgene expression due to a counter regulation within the cell and/or a diminished number of available proteins due to the self-oligomerization of dnASC molecules are possible facts for the observed phenotype and should be considered in future experiments (Bryan et al. 2009; de 2009; Liepinsh et al. 2003; Lu et al. 2014).

In parallel, another vector encoding an additional GFP overexpression enabling flow cytometry-based sorting was tested. Unfortunately, this vector did not allow inducible expression. Upon viral infection of DCs, the number of dead cells dramatically augmented over time possibly referring to a dnASC-self-oligomerization-induced cell

death (Bryan et al. 2009; de 2009; Hasegawa et al. 2007; Liepinsh et al. 2003; Lu et al. 2014; Masumoto et al. 2001).

Functional experiments were performed with sorted living and GFP/dnASC expressing DCs. Only in 2 out of 4 experiments the cell number after sorting was sufficient for subsequent functional analysis of dnASC-mediated inflammasome inhibition. The dnASC expression led to diminished IL-1β secretion upon ATP stimulation in mature DCs in only one of these experiments, leaving my hypothesis still open (Figure 18) (Johnston et al. 2005; Johnston et al. 2007; Martinon et al. 2002). Unfortunately, in the second experiment, the ATP stimulation did not lead to IL-1β secretion of control vector infected cells. Future repetition experiments are indispensable and needed to analyse the impact of a dnASC expression on the IL-1β secretion in DCs.

The development of an inducible siRNA-mediated knockdown model system in parallel could enable a comparison between different strategies used by some pathogens (Liau et al. 2006; Patzel et al. 2005; Szulc et al. 2006). No conclusion about the inner cellular ASC expression can be made in the used experimental system. Another antibody recognizing only the PYD-domain should be developed for characterisation of protein expression (also for the caspases), and if new tools are available, protein expression in the inflammatory signalling cascade especially for the adaptor molecules like ASC and the caspases in DCs should be re-examined. Summarizing my data, the approach from Martinon, Burns and Tschopp using the over-expression of the dominant-negative version of ASC is experimentally limited in primary DCs (Martinon et al. 2002).

Furthermore, I hypothesize that a disturbance of the signalling cascade by expression of intracellular proteins like dnASC leading to accumulation / self-oligomerization and finally to the activation of cell death pathways in DCs could be a benefit for the resistance of a pathogen. Additionally, the reduced cytokine secretion upon infection due to inflammasome inhibition reducing the immune response and/or the direction of the immune response is another advantage for survival of the pathogen. All these different aspects would need further investigation for characterisation of host – pathogen interactions.

4.1.5. The inflammatory impact on T cell proliferation

To complete the IL-1β life cycle and functional chain, I next investigated the impact of IL-1β on TC activation in the CD4+ TC-DC interaction model system. Especially the cytokine panel after cell-cell interaction determining the direction of immune response and CD4+ TC differentiation as well as cell death during interaction was analysed in the model system used here. Affirmed by my data the cytokine IL-1β alone does not lead to TC activation (Figure 19); a further costimulatory signal mediated by a cell-cell contact is needed (Caux et al. 1994; Gonzalo et al. 2001; Inaba et al. 1994).

It has been demonstrated that IL-1 (α and β) and IL-23 have a crucial role in the induction of IL-17 producing T cells (Besnard et al. 2011; Sutton et al. 2006; Zheng et al. 2007). Anticipating the direction of the immune response in my model system mainly into Th17, I found a more complex cytokine profile upon interaction of LPS-matured and $P2X_7$-stimulated DCs with CD4+ TCs (Figure 20). The detected cytokines secreted during interaction in my experimental system were TNF-α, IL-2, IL-4, IL-10, GM-CSF, IFN-γ and G-CSF. High levels of M-CSF and IL-5 were found in supernatants independent on DC activation. In one experiment IL-1α, IL-6 and IL-17 were also analysed, showing a secretion of IL-6 and IL-17 upon DC-TC interaction (data not shown). These results indicate for an ambiguous immune response into Th1 (IL-2, IFN-γ, IL-12, TNF-β, GM-CSF), Th2 (IL-4, IL-10, IL-5, IL-6, IL-13), Th22 (TNF-α, IL-22) and Th17 (IL-17, IL21, IL22, IL24, IL26, GM-CSF) upon LPS maturation and argue for a more complex immune reaction depending on the infection background.

So, probably a mixture of pathogenic molecules limits and drives the immune response finally into a specific direction (Becher and Segal 2011; Codarri et al. 2011; Constant et al. 1995; El-Behi et al. 2011; Iezzi et al. 1999; Jonuleit et al. 2001; Kuchroo et al. 1995; Langenkamp et al. 2000). Furthermore, an imbalance in TC responses like shown for Th1/Th2 and Th17/Treg is central for pathogenesis of asthma and probably other inflammatory diseases (Cosmi et al. 2011; Dechene 2002; Jiang et al. 2015b; Kim et al. 2010; Li et al. 2018; Shi and Qin 2005).

Due to the fact that DCs are infected with lentivirus, the CD8+ TC differentiation after inflammasome activation in the interaction model system should be additionally analysed in future experiments especially in respect to the cytokine profile.

4.2. Insights into the CD95L- mediated apoptotic signalling pathway in DCs

Upon activation and induction of a maximal immune response programmed cell death is initialized in DCs by intrinsic, extrinsic and T-cell mediated signals, which is essential for their life cycle regulation (Kushwah and Hu 2010). DC-apoptosis is important for regulation of the equilibrium between tolerance and immunity through many different pathways (Kushwah and Hu 2010; Wong et al. 1997). Thereby, the magnitude of immune response is regulated because the availability of antigen for TCs is limited. Defects in DCs apoptosis have been associated with different pathologies like sepsis, breast cancer and autoimmune diseases (Chen et al. 2006c; Ito et al. 2006; Pinzon-Charry et al. 2007). Whereas the exact genes that regulate DC lifespan have not been fully determined, some other pathways were shown to be involved in the control of DCs survival (Lehner et al. 2012; Leverkus et al. 2000b; Leverkus et al. 2003a; McLellan et al. 2000; Wang et al. 2009).

In this thesis, I focussed on the best studied CD95L-mediated cell death pathway (Krammer 2000). Upon binding of CD95L to the receptor CD95 and its trimerization (Schulze-Osthoff et al. 1998), FADD and subsequently the inactive (initiator) procaspase-8 was recruited (Kischkel et al. 1995; Muzio et al. 1996). One of the central regulators of caspase-8 function is the procaspase-8-like, protease-deficient homolog of caspase-8 named cFLIP (Irmler et al. 1997). In my thesis, the role of cFLIP in the control of apoptosis and function during the lifespan of primary DCs was explored.

I first established that murine DC rapidly upregulate cFLIP upon LPS-induced maturation at the mRNA level, whereas caspase-8 levels remained largely unchanged (Fig. 21). These data argue that maturation tilts the stoichiometry between caspase-8 and cFLIP towards cFLIP, conferring resistance to death receptor-mediated cell death. Analysis of the caspase-8 activity of DC following death receptor stimulation in either immature or mature DC strongly argue that DC maturation blocks apoptotic caspase activation by cFLIP expression, as I detected a robust cleavage of caspase-8 to the active p18 fragment only in immature DC (Fig. 22).

During immune responses, elimination of immature DC encourages the most fitting DC to reach the lymph node and present antigens. The killing of immature DC by NK cells for example seems to be largely dependent on death receptor signalling pathways (Hayakawa et al. 2004). Interestingly, I also detected a release of the high mobility group box 1 protein (HMGB-1) into the supernatant of immature DCs upon CD95L stimulation (data not shown) arguing for an additional activation of a necrotic form of cell death (Scaffidi et al. 2002). Recent studies have shown that the HMGB1 as one important DAMP may also contribute to DC maturation by binding to TLR2 and TLR4 (Fitzgerald et al. 2001) and additionally probably stimulates DC to secrete more cytokines and pro-inflammatory mediators to help characterize an innate inflammatory response like shown for macrophages (Altemeier et al. 2007; de Oliveira et al. 2007; Messmer et al. 2004; Neff et al. 2005; Park et al. 2003; Saenz et al. 2014; Vande et al. 2011; Yang et al. 2007). Further studies on the role of the CD95 signalling pathway and the HMGB1 release in inflammation and apoptosis in immune responses especially for DCs would lead to an improvement of immunotherapies (Jiang et al. 2015a).

CD95 receptor mediated apoptosis requires the formation of the death inducing signalling complex (DISC), which contains the adaptor molecule FADD, cFLIP and caspase-8 (Feoktistova et al. 2011; Geserick et al. 2009; Kischkel et al. 1995; Muzio et al. 1996; Scaffidi et al. 1999b). Mice constitutively lacking caspase-8, cFLIP, or FADD have demonstrated the critical role of these molecules in embryonic development and precluded the cell-type specific role of these molecules (Varfolomeev et al. 1998; Yeh et al. 2000). My data using a CD95L fusion protein to perform ligand affinity precipitation of the murine CD95 DISC demonstrated, that the DISC rapidly forms, that cFLIP was highly enriched in the DISC with high affinity, and that caspase-8 was cleaved and thus activated within the DISC. I detected for the first time the DISC formation in primary murine DCs (Figure 23). First, I was able to detect the core proteins of the DISC in both immature and mature DCs. Moreover, whenever cell death was inhibited as exemplified by the cell death-resistant mature DC, the important contribution of caspase-8 for a negative regulation has been elucidated in the last years (Kaiser et al. 2011; Oberst et al. 2011). Thus, future studies will most likely elucidate the function of surviving DC and in particular the impact of death receptor signalling beyond apoptotic caspase activation in more

detail. It is perceivable that a limited induction of active caspase-8 in the DISC or further downstream complexes named complex II (Lavrik and Krammer 2012) or Ripoptosome (Feoktistova et al. 2011; Tenev et al. 2011) is heavily regulated by the isoform distribution or expression level of cFLIP. As DCs upregulate $cFLIP_L$ as well as $cFLIP_R$, the functional outcome to this point is rather unclear and warrants further study. Recent studies have shown that overexpression of $cFLIP_R$ in hematopoietic cells protects lymphocytes against CD95-induced apoptosis and activation-induced cell death (Telieps et al. 2013) but its permanent expression as well as the expression of the baculoviral caspase inhibitor (p35) in DCs leads later to autoimmunity (Chen et al. 2006c; Ewald et al. 2014).

To date, the functional relevance of cFLIP for DC has been unclear, as the prior studies solely correlated cell death sensitivity and cFLIP levels. I thus tested the hypothesis that cFLIP expression impacts DC survival more directly by selective knockout of cFLIP from DC. I used two different approaches for the elimination of cFLIP in primary DC: At first, I determined whether genetic deletion of the cFLIP locus *in vitro* would modulate DC function and cell death sensitivity, and second I downregulated cFLIP expression in an inducible manner using a lentiviral approach.

Taking advantage of BMDC generated from mice carrying the loxP FLIP allele (Zhang and He 2005), I deleted the cFLIP locus using a Cre-expressing lentiviral approach as published (Heldt and Ressler 2009; Panayotova-Dimitrova et al. 2013; Vince et al. 2007). Supporting the notion that cFLIP is critical for survival of mature BMDC, elimination of both cFLIP alleles in primary DCs led to spontaneous cell death, which was rescued by the presence of at least one of the cFLIP alleles (Figure 24). These data demonstrated the tremendous impact of cFLIP in the control of the programmed cell death in DCs but precluded a more thorough analysis of DC function whenever cFLIP is absent. I thus turned my attention to a less stringent elimination of cFLIP using inducible lentiviral expression of a shRNA against cFLIP. To test whether down-regulation of cFLIP will further influence the viability and functionality of DC, I used the LV-mediated siRNA silencing in BMDCs. I demonstrated that upon cFLIP down-regulation BMDCs were sensitized to CD95-induced cell death in a caspase-dependent manner (Figure 25). In contrast to an overexpression of vFLIP in mice enhancing Th1 and Th2 cytokine response upon *Leishmania major* infection (Pereira-Manfro et al. 2014), I hypothesized that in a

cFLIP knockdown situation the T cell response is diminished as well as the number of viable DCs during time of cell interaction. Despite the highly reduced amount of cFLIP in those cells, I was unable to detect significant effects neither on their capacity to stimulate T cells in vitro nor on DC cell number (Figure 26). Already the priming of DCs with ovalbumin led to TC stimulation upon cell interaction independent of the cFLIP level in DCs and somehow stabilized DCs lifespan. However, I suggest that a diminished cFLIP expression in DCs probably influences the direction of the immune response. To what extend the generated knockdown data are valid still remains open and demands other experimental systems for further specific functional analysis in primary DCs.

DC apoptosis and its modulation have been recently related to the therapeutic implication of dendritic cells. Clinical trials using DC-based immunotherapy however have not been very promising (Nencioni et al. 2008). It was shown that less than 10% of injected DCs during DC therapy migrate to lymph nodes, and not much is known about the fate of the remaining DCs (Josien et al. 2000; Schuler et al. 2003). One possible explanation could be that a major amount of DCs die by apoptosis upon injection and are rapidly taken up by viable DCs, resulting in limited induction of protective antitumor immune response and tolerance (Kushwah et al. 2009; Kushwah et al. 2010; Kushwah and Hu 2010). It has thus been suggested that DCs with increased cell death resistance could improve DC immunotherapy by limiting DC programmed cell death. Recent studies reported different approaches to achieve this task, like siRNA targeting approaches against pro-apoptotic molecules (Kim et al. 2009; Wang et al. 2009) or the treatment of DCs with DC prosurvival factors (Escribano et al. 2009; Mattioli et al. 2009). Lehner and authors have recently shown the importance of autocrine TNF to protect DC from cell death induction (Figure 2 and 3) by regulating BAK, BCL-2 and $cFLIP_L$ (Lehner et al. 2012). Beside Bcl-2, $cFLIP_L$ is a major protection factor that inhibits TNF-induced cell death. It is perceivable that the upregulation of $cFLIP_L$, or consequently a critical lower threshold of $cFLIP_L$ is of critical importance to protect DC from TNF-induced cell death.

Understanding how the programmed cell death control is regulated in both mature and immature DCs, would give further information about the mechanisms of inhibition of cell death, and this information will help to further develop strategies that may result in improvement of DC-based therapeutics. Beyond demonstrating the

significant role of $cFLIP_L$ in the control of the programmed cell death, my data now suggest $cFLIP_L$ as potential target molecule in triggering DC apoptosis in the future attempt to improve the DC-based immunotherapy.

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6. Appendix

6.1. Abbreviations

% (v/v)	percent by volume
% (w/v)	percent by mass
4-HT	4-Hydroxytamoxifen
Aa	amino acids
AIF	Apoptosis-Inducing Factor
AP-1	Activating Protein 1
APC	antigen presenting cell
ATP	Adenosin-Tri-Phosphate
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-Xl	B-cell lymphoma-extra large
BID	BH3 interacting domain death agonist
BIM	Bcl-2 interacting mediator of cell death
BIR	Baculoviral IAP Repeat
BM	bone marrow
BMF	Bcl-2-modifying factor
CAD	Caspase dependent desoxyribonuclease
CARD	Caspase Activation and Recruitment Domain
Caspase	Cysteinyl-Aspartate Specific Protease
CD95	FasL (FS7-associated cell surface antigen)
CD95L	FasL (FS7-associated cell surface antigen Ligand)
cFLIP	cellular FLICE-Inhibitory Protein
cIAP	cellular Inhibitor of Apoptosis Protein
CIAP	Alkaline Phosphatase from calf intestine
CICD	Caspase-independent cell death
CDCD	Caspase-dependent cell death
CM	Cleavage mutant
COP	CARD only protein
CTL	cytotoxic T lymphocyte
DAMP	danger-associated molecular pattern
DC	dendritic cell
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
D-MEM	Dulbecco´s Modified Eagle Medium
Dn	dominant negative
DNA	deoxyribonucleic acid
dNTPs	Deoxynucleoside Triphosphate Set
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
Expo	exposition
F	Flag (tag)
FACS	fluorescence activated cell sorter
FADD	Fas-Associated Death Domain protein
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable
Fig	Figure
FLICE	FADD-like interleukin-1 beta-converting enzyme
GFP	Green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
HA	Hyaluronic acid
HaCaT	human adult low calcium temperature (keratinocytes)
HEK 293T	Human Embryonic Kidney 293 large T transformed
HMGB1	High-Mobility-Group-Protein B1
HRP	Horseradish peroxidase
HepS	Heparansulfate
HRS	Hyper random sequence
HHV-8	Human herpesvirus 8
HVS8	herpesvirus saimiri 8
IAP	inhibitor of apoptosis
IFN	Interferon
IgG	Immunoglobulin G
IκB	Inhibitor of NF-κB
IKK	Inhibitor of κB Kinase
IL	Interleukin
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
K	lysine
KD	Kinase Dead
kDa	kilo Dalton
LA	Lipoteichonic acid
LPS	Lipopolysaccharide
LRR	leucine rich repeat
Mal	MyD88-adapter-like
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage colony-stimulating factor
MHC	major histocompatibility complex
MDP	muramyldipeptide
MWS	Muckle-Wells-syndrome
MW	Molecular Weight
MyD88	Myeloid differentiation primary response gene (88)
NACHT	NAIP (neuronal apoptosis inhibitor protein), C2TA [class 2 transcription activator, of the MHC, HET-E (heterokaryon incompatibility) and TP1 (telomerase-associated protein 1)
NALP	NLRP [Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing]
NEMO	NF-κB Essential Modulator
NOMID	Neonatal Onset Multisystem Inflammatory Disease
NF-κB	Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells

NLR	NOD – like receptor family
NOD	nucleotide-binding and oligomerization domain
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PGN	Peptidoglycan
PI	Propidium Iodide
POP	PYD only protein
PRR	PAMP recognition receptors
PVDF	Polyvinylidene fluoride
PYD	Pyrin domain
QVD	(Q-VD-OPH) Q-Val-Asp(non-omethylated)-OPh
R	Receptor
RIP1	Receptor-Interacting Protein 1
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of Mean
ShRNA	Small hairpin RNA
SiRNA	Small interfering RNA
SMAC/DIABLO	Second Mitochondrial Activator of Caspases/Direct IAP Binding protein with Low pI
TC	T cell
TAE	Tris-acetate-EDTA
TAK1	Transforming Growth Factor-β (TGF-β)-Activated Kinase 1
Th	T helper
TIR	Toll/IL-1 receptor domain
TL	Total Lysate
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
U	Unit
UV	Ultra Violet
vFLIP	viral FLICE-Inhibitory Protein
WB	western blot
WT	Wild type
ZVAD-fmk	z-Val-Ala-DL-Asp(OMe)-fluoromethylketone

7. Ehrenerklärung

Ich versichere hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; verwendete fremde und eigene Quellen sind als solche kenntlich gemacht.

Ich habe insbesondere nicht wissentlich:

- Ergebnisse erfunden oder widersprüchlich Ergebnisse verschwiegen,
- statistische Verfahren absichtlich missbraucht, um Daten in ungerechtfertigter Weise zu interpretieren,
- fremde Ergebnisse oder Veröffentlichungen plagiiert,
- fremde Forschungsergebnisse verzerrt wiedergegeben.

Mir ist bekannt, dass Verstöße gegen das Urheberrecht Unterlassungs- und Schadensersatzansprüche des Urhebers sowie eine strafrechtliche Ahndung durch die Strafverfolgungsbehörden begründen kann.

Ich erkläre mich damit einverstanden, dass die Arbeit ggf. mit Mitteln der elektronischen Datenverarbeitung auf Plagiate überprüft werden kann.

Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form als Dissertation eingereicht und ist als Ganzes auch noch nicht veröffentlicht.

Gatersleben, der 27.05.2019

Diplom Biologin Beate Fraust