A Systematical Analysis of the Signal Transduction Pathways Induced by the Latent Membrane Protein 1 (LMP1) of Epstein-Barr Virus

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1 INTRODUCTION

1.1 The Epstein-Barr Virus

In 1964 Anthony Epstein and his colleagues Yvonne Barr and Bert Achong discovered a novel virus particle in cell culture samples from African Burkitt's Lymphoma (Epstein et al, 1964). The new virus was later termed Epstein-Barr virus (EBV, alternatively human herpesvirus 4, HHV-4) and turned out to be a member of the gamma-1 herpesviruses, which are also called lymphocryptoviruses and solely infect primates. These double stranded DNA viruses are characterized by their ability to persist life-long in an infected individual by switching their genetic program from a lytic to a latency program. During latency no progeny virus is produced and the virus resides hidden in long-lived memory B cells (Amon & Farrell, 2005; Kuppers, 2003).

EBV is highly prevalent with over 90 % of the adult population world-wide carrying the virus (Cohen, 2000; Evans, 1972). When a person gets exposed to EBV during childhood, the infection is mostly asymptomatic or resembles other acute, harmless virus infections. EBV infection during young adulthood, on the other hand, can lead to a distinct pathological condition called infectious mononucleosis (IM), which is a self-limiting lymphoproliferative disease (Henle et al, 1968; Valachis & Kofteridis, 2012). Even so, EBV infection is also associated with several malignant conditions (Kutok & Wang, 2006). Especially EBV-positive individuals suffering from genetic or acquired immunocompromised conditions, like organ-transplant patients or patients

with acquired immunodeficiency syndrome (AIDS), can develop serious complications and are more prone to malignant lymphoproliferative diseases associated with EBV (Cesarman, 2011; Evans, 1972).

1.1.1 Mechanisms of Infection and Persistence

EBV infects epithelial cells of the oral cavity and respiratory tract as well as resting primary B cells, in which it can establish life-long persistency (Cohen, 2000; Thorley-Lawson et al, 2008). The receptor for EBV on B cells is the cell surface protein CD21, which is recognized by the viral envelope protein gp350/220 (BLLF1), followed by internalization of the virus into the cell (Carel et al, 1990; Fingeroth et al, 1984; Nemerow & Cooper, 1984). It is so far unknown how EBV gains access into cells not expressing CD21, but studies showing successful infection of B cells and epithelial cells by EBV particles lacking gp350/220 suggest that alternative pathways of entry must exist (Janz et al, 2000). Distinct sets of viral genes define either a lytic or a latent phase of EBV's life cycle. During lytic replication, the vast majority of viral genes (the lytic genes) are transcribed to form new progeny virus (Tsurumi et al, 2005). Upon infection, EBV establishes latency in the target cells (Kalla & Hammerschmidt, 2012). The 184 kB linear viral DNA forms a circular episome (Kaschka-Dierich et al, 1976; Lindahl et al, 1976) that is maintained by the cellular replication machinery (Dhar et al, 2001; Ritzi et al, 2003) and persists in a latent state for the lifetime of the cell or until another lytic replication cycle is induced (Kutok & Wang, 2006). Four different latency programs are known for EBV, which are each characterized by the transcription of a strictly limited number of latent genes and are summarized in table 1-1 (Klein et al, 2010; Niedobitek et al, 2001). Among the latent gene products are 6 EBV-encoded nuclear antigens (EBNA1, 2, 3A, 3B, 3C, LP), two nonpolyadenylated RNAs (EBV-encoded small RNA (EBER) 1 and 2), and three latent membrane proteins (LMP1, 2A and 2B) (Kuppers, 2003).

All of these latently expressed proteins are important either for persistence of the EBV genome or for the transformation of cells by driving proliferation, influencing the cell cycle, or interfering with apoptosis. For example, EBNA1 is essential for episomal maintenance and segregation (Frappier, 2012; Lee et al, 1999), was shown to contribute to B cell immortalization by enhancing the transcription of EBV's transforming genes (Altmann et al, 2006), and plays a role in immunoevasion, because it cannot be processed for MHC-I-presentation due to several Gly-Ala repeats (Levitskaya et al, 1997). EBNA2 and 3C play a role in cell transformation by transactivating for instance LMP1 (Wang et al, 1990; Zhao & Sample, 2000), and LMP2A can rescue B cells with a crippled B cell receptor from apoptosis by mimicking the B cell receptor function and granting pro-survival signals (Caldwell et al, 1998; Mancao & Hammerschmidt, 2007; Merchant et al, 2000). LMP1, which is the primary oncogene of EBV, will be extensively described in chapter 1.2.

latency type	gene products expressed	related malignancies/cell types
0	EBERs	memory B-cells
I	EBERs, EBNA1	BL
II	EBERs, EBNA1, LMP1, LMP2A, LMP2B	HL, NPC
III	EBERs, all EBNAs, LMP1, LMP2A, LMP2B	PTLD, IM, LCLs

Table 1-1. Latency types of EBV

1.1.2 EBV and its Association with Malignant Diseases

In a healthy individual, the immune system normally keeps EBV infection under surveillance and eliminates most cells expressing the latent proteins, to maintain the asymptomatic, healthy state of latent infection (Callan et al, 1998; Zhang et al, 2012). Nonetheless, since its discovery EBV has been found to be associated with numerous pathological conditions and cancers (Cohen, 2000; Kutok & Wang, 2006; Niedobitek et al, 2001; Okano, 1998), and has therefore been classified a class 1 oncogene by the world health organization (WHO) (1997 IARC monograph). In vitro, the oncogenic potential of EBV was demonstrated by successfully immortalizing primary B lymphocytes with the virus (Pope et al, 1973). This gives rise to continuously growing lymphoblastoid cell lines (LCLs), which express a latency type III pattern of genes.

In case of a compromised immune system, uncontrolled cell proliferation driven by latently expressed proteins can occur (Zhang et al, 2012). This can be seen in diseases like posttransplant lymphoproliferative disorder (PTLD), which mostly displays a latency type III phenotype (Kutok & Wang, 2006; Loren et al, 2003). The potential driving force of EBV in these diseases was shown by transplantation of EBV-immortalized B cells into immunodeficient SCID mice, which resulted in rapid tumor formation (Rowe et al, 1991). Although immunocompromised patients are more prone to EBV-related diseases, the virus can also be found associated with malignant diseases affecting immunocompetent patients, of which Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL) or nasopharyngeal carcinoma (NPC) are the most frequent.

Burkitt's lymphoma is characterized by deregulated c-myc expression following *c-MYC* translocation. EBV expressing a latency type I program is frequently found in BL cells. Although EBV can be found in a large proportion of BL, it seems not to be essential for BL development,

but might rather provide advantageous proliferative effects (Bornkamm, 2009; Molyneux et al, 2012; Thorley-Lawson & Allday, 2008).

A type II latency program is found in EBV positive Hodgkin's lymphoma. Here, elevated NF-κB activity, which can result from expression of EBV latent proteins, possibly rescues B cells with faulty Ig rearrangements during somatic hypermutation from apoptosis (Kuppers, 2003). This gives rise to malignant Hodgkin/Reed-Sternberg (HRS) cells (Kuppers et al, 1998), which are responsible for uncontrolled, chronic inflammation. The severity of inflammation is often EBV-related (Herbst et al, 1997) and attracts a vast number of inflammatory cells, which make up the bulk of HL-associated cells (Kuppers, 2009; Kuppers et al, 2012).

Undifferentiated nasopharyngeal carcinoma is a tumor of nasopharyngeal epithelial cells, which is especially prevalent in Southeast Asia and North Africa, and which is strongly related to EBV infection (Burgos, 2005; Klein et al, 1974; Nicholls et al, 1997; Niedobitek et al, 1991). The fact that EBV can be detected in early, undifferentiated lesions of NPC (Pathmanathan et al, 1995), and that the EBV genome found in established tumors is monoclonal (Raab-Traub & Flynn, 1986), implies that the virus is a driving force of NPC development. While EBNA-1 and LMP2A can be detected in virtually all EBV-positive NPC samples, LMP1 seems to be more important for early, preinvasive lesions that drive neoplasia and tumor formation (Brooks et al, 1992; Niedobitek et al, 1992; Pathmanathan et al, 1995). However, the frequency of LMP1 in NPC samples varies among different studies, which might be attributed to the sensitivity of the applied detection methods (Tsao et al, 2002). A very high association of LMP1 expression with NPC was demonstrated by detecting LMP1 in nasopharyngeal swabs of over 90% of NPC patients by RT-PCR methods (Lin et al, 2001). Generally, the deregulation of several cellular signaling pathways by LMP1 can contribute greatly to the progression of NPC (Dawson et al, 2012; Zheng et al, 2007). Like many other tumors, NPC is accompanied by heavy chronic inflammation that adds to the severity of the condition. Several studies have shown that the high upregulation of pro-inflammatory cytokines like IL-1α/β, CXCL1,GM-CSF, IL-6 or IL-8 in NPC seems to be related to EBV infection and LMP1 expression (Hannigan et al, 2011; Huang et al, 1999; Lai et al, 2010; Li et al, 2007; Morris et al, 2008). LMP1 signaling is furthermore linked to angiogenesis in NPC (Yoshizaki et al, 2001), and it was shown that LMP1 is helping NPC-cells to evade TRAIL-induced apoptosis (Li et al, 2011) or to promote metastatic growth (Chew et al, 2010; Yoshizaki, 2002).

In summary, although EBV infection is not consistently found in all of these tumors, and other environmental factors contribute to neoplasia, the oncogenic potential of the virus is evident. EBV can aid tumor formation or provide growth advantage to forming neoplastic cells through latently expressed proteins (Knecht et al, 2001).

1.2 Latent Membrane Protein 1 (LMP1)

Latent membrane protein 1 (LMP1) is expressed during EBV latency programs II and III. It was the first of the EBV latent proteins to be shown to exert transforming potential on its own. Rodent fibroblasts transfected with LMP1 alone display phenotypic changes associated with transformation, like focus formation or serum independent cell growth, and are tumorigenic in nude mice (Moorthy & Thorley-Lawson, 1993; Wang et al, 1985). Furthermore, EBV lacking functional LMP1 is not capable of efficiently transforming B cells (Kaye et al, 1993). Several studies have shown that LMP1 plays an essential part in the successful growth transformation of B lymphocytes by mimicking CD40 signals (Dirmeier et al, 2003; Kilger et al, 1998), which proves that LMP1 is the primary oncogene of EBV. To back that, in vivo studies demonstrated that transgenic mice, which express LMP1 in the B cell compartment, developed lymphoma significantly more often than control animals (Kulwichit et al, 1998; Zhang et al, 2012).

The biological activity of LMP1 contributes to cell transformation and malignant growth through different mechanisms. Expression of LMP1 can confer resistance to apoptosis by upregulation of pro-survival proteins like Bcl-2, A20 or Mcl-1, but also by the inhibition of the pro-apoptotic protein Bax (Grimm et al, 2005; Henderson et al, 1991; Laherty et al, 1992; Wang et al, 1996). Furthermore, promotion of proliferation, cell growth and cell cycle progression can be attributed to LMP1's ability to induce the expression of c-myc or the epidermal growth factor receptor (EGFR), and to upregulate Cdc2 (Dirmeier et al, 2005; Kutz et al, 2008; Miller et al, 1995). LMP1 expression is also closely linked to malignant tumor progression. LMP1 signaling can induce cytokines and growth factors such as Interleukin (IL) -1, IL-6, IL-8, IL-10, VEGF[1], CXCL1[2], CXCR4[3] or GM-CSF[4], which are involved in promoting angiogenesis and inflammation (Eliopoulos et al, 1997; Hannigan et al, 2011; Huang et al, 1999; Lambert & Martinez, 2007; Morris et al, 2008; Murono et al, 2001; Yoshizaki et al, 2001). Additionally, LMP1 is capable of upregulating the matrix metalloprotease (MMP) 9, which can aid metastasis and invasion (Stevenson et al, 2005; Yoshizaki et al, 1998). Most importantly, LMP1 induces several cellular signaling pathways that are critical for cell cycle control, survival and proliferation, such as the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathways, the PI3K/Akt (phosphatidylinositide 3-kinase/protein kinase B (PKB)) pathway, the JAK (janus kinase)/STAT

[1] VEGF (vascular endothelial growth factor), cytokine that stimulates angiogenesis
[2] CXCL1 (CXC chemokine ligand 1, GRO1, KC) mitogenic chemokine involved in inflammation, angiogenesis and tumorigenesis
[3] CXCR4 (CXC chemokine receptor 4, CD184), chemokine receptor important for T-cell homing, associated with cancer and metastasis
[4] GM-CSF (ganulocyte and macrophage colony stimulating factor, CSF-2), cytokine involved in lineage commitment of granulocytes and macrophages, and in inflammation

(signal transducer and activator of transcription) pathway, or MAPK (mitogen activated protein kinase) pathways (Eliopoulos & Young, 2001; Kieser, 2007), which will all be extensively described later (chapter 1.6).

1.2.1 Structural Features of LMP1

The LMP1 protein, which is encoded by the EBV-gene *BNLF1*, is a 63 kDa protein of 386 amino acids (aa) and can be divided into three major domains: 1) a short intracellular N-terminal domain (aa 1 – 24), 2) six transmembrane helices (TM1-6, aa 25 - 186) that span the cellular membrane, and 3) a cytoplasmic C-terminal domain (aa 187 - 386) that harbors LMP1's signaling activating regions (Figure 1-1). All three domains are important for the transforming properties of LMP1 (Moorthy & Thorley-Lawson, 1993). It was shown that the N-terminus does not employ any biological activity that helps transformation, but it is important for the localization of LMP1 inside the plasma membrane as well as protein turnover (Aviel et al, 2000; Coffin et al, 2001; Izumi et al, 1994). LMP1 activity is independent of a ligand, but requires oligomerization of LMP1, which is mediated by the transmembrane domain (Coffin et al, 2001; Floettmann & Rowe, 1997; Gires et al, 1997). Fusion proteins between the LMP1 hydrophobic 6-helix domain and the signaling domain of CD40, TNFR1 or TNFR2 resulted in a constitutively active receptor, which shows that LMP1's transmembrane domain is sufficient to oligomerize in the absence of a ligand (Gires et al, 1997; Hatzivassiliou et al, 1998; Schneider et al, 2008). The C-terminal cytoplasmic domain of LMP1 conveys the molecular signaling properties of the protein that are necessary for cellular transformation. Two major subdomains were identified through mutational analyses: C-terminal activating regions (CTAR) 1 and 2 (Brodeur et al, 1997; Huen et al, 1995). Both sub-domains are involved in recruiting cellular signaling molecules in order to trigger a broad range of signaling pathways, which will be described in more detail later.

CTAR1 spans amino acids 194 – 232 of LMP1 and is characterized by the sequence P_{204}QQAT. This site contains the conserved consensus motif PxQxT/S, which is the core binding site for cellular adapter proteins of the family of tumor necrosis factor receptor-associated factors (TRAFs) (Ha et al, 2009). CTAR1 is able to associate with TRAF1, TRAF2, TRAF3 and TRAF5, but not TRAF6 (Brodeur et al, 1997; Devergne et al, 1996; Mosialos et al, 1995; Sandberg et al, 1997), and interaction studies with in vitro translated TRAF proteins showed that TRAF1, TRAF2, and TRAF3 can bind directly to P_{204}QQAT (Devergne et al, 1996). Mutation of this site to A_{204}xAxT is sufficient to inhibit any association of TRAF1 or TRAF2 with LMP1-CTAR1 (Devergne et al, 1996). While the same mutation abolishes TRAF3-binding to a short LMP1-peptide (aa 199-214), it is not sufficient in the context of a longer sequence encompassing all of CTAR1 (aa 187-231), showing that other residues outside the core motif are involved in TRAF3-binding (Devergne et al, 1996). Recently, one study has implied that TRAF6 may also bind to the

TRAF-binding motif in CTAR1, by blocking co-precipitation of LMP1 and TRAF6 through mutation of the TRAF-binding site to A_{204}xAxT (Arcipowski et al, 2011). However, this observation has not been made by any other laboratories so far, including ours.

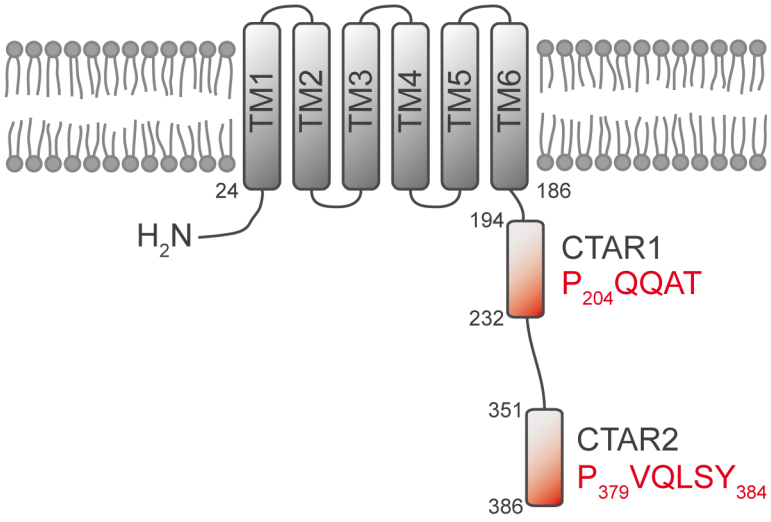

Figure 1-1. Schematic overview of the LMP1 protein. LMP1 is anchored in the plasma membrane by its six transmembrane domains (TM1-6). Both the N- and C-terminus are cytoplasmic. The C-terminal tail is crucial for LMP1 signaling activity, because it harbors LMP1's two major signaling activation regions CTAR1 and CTAR2. A PxQxT motif in CTAR1 is capable of binding TRAF molecules, and the sequence PVQLSY was shown to be essential for activation of signaling pathways by CTAR2. Point mutations of either site (A_{204}xAxA or Y_{384}G) drastically reduce the potential of LMP1 to transform B-cells (Dirmeier et al, 2003).

CTAR2 is located at the far C-terminal end of LMP1 (aa 351 – 386). The sequence P_{379}VQLSY$_{384}$ within CTAR2 was mapped to be the core sequence responsible for interaction of CTAR2 with cellular effector proteins and for activation of signaling pathways (Floettmann & Rowe, 1997; Izumi & Kieff, 1997; Kieser et al, 1999; Kieser et al, 1997). The first protein described to bind directly to CTAR2 in a yeast-two-hybrid screening was the tumor necrosis factor receptor associated death domain protein (TRADD) (Izumi et al, 1999b). Although a motif resembling the PxQxT/S TRAF-binding sequence is included in CTAR2, the direct interaction of CTAR2 with any TRAF-protein has never been demonstrated. It was suggested that BS69 could serve as a mediator of TRAF6-recruitment to CTAR2 (Wan et al, 2006). Only very recent work of our laboratory has provided proof that TRAF6 but not TRAF2 does in fact directly bind to the P_{379}VQLSY motif of CTAR2 (Giehler, 2012).

The region between CTAR1 and CTAR2 is not essential for B cell transformation. LMP1 lacking aa 232 – 351 displayed the same transformational properties as wildtype (wt) LMP1, and was similarly capable of recruiting cellular factors such as TRAFs and TRADD (Izumi et al, 1999a). Nevertheless, the same region contains two proline-rich box1 regions (P_{275}HDPLP and P_{302}HDPLP) that were described to be binding sites for members of the Janus kinase family (JAK), and LMP1 can recruit JAK3 to this region (termed CTAR3) (Gires et al, 1999).

1.3 TNF-Receptor Associated Factors – the TRAF Family

Like the members of the TNFR family, LMP1 lacks intrinsic enzymatic activitiy and therefore relies on adaptor molecules like TRAFs to induce cellular signaling cascades.

The family of TNFR-associated factors (TRAFs) consists of seven members in mammals, TRAF 1 – 7. They are vitally important adaptor molecules for a wide array of signaling cascades and cellular functions, and have been associated with receptors of the TNFR family, the Toll-like receptor/IL-1 receptor (TIR) family and several viral receptors like LMP1, among others (Chung et al, 2002; Ha et al, 2009; Xie et al, 2008). With the exception of TRAF7, all TRAFs are characterized by a highly conserved C-terminal TRAF domain (Ha et al, 2009; Rothe et al, 1994). This domain comprises a coiled-coil domain and a TRAF-C domain, which promotes homo- and hetero-oligomerization and tethers TRAFs to the cytoplasmic regions of receptors (Park et al, 1999; Takeuchi et al, 1996; Ye et al, 1999). Instead of a TRAF-domain, the C-terminus of TRAF7 harbors seven WD40 repeats that facilitate interaction with MEKK3 (Xu et al, 2004). The N-terminal part of TRAFs 2-7 further harbors a RING (really interesting new gene)-domain followed by several zinc-finger motifs (Rothe et al, 1994). The RING-domain is important for the enzymatic function of TRAFs, as it operates as an E3-ubiquitin ligase (Deng et al, 2000). Ubiquitination, like phosphorylation, offers an effective mechanism to alter the functional impact of a protein on its environment and on cellular signaling pathways. Next to K48-linked ubiquitin chains, which target proteins to proteasomal degradation, other modes of ubiquitination, like K63-linked ubiquitination, are known. These K63-ubiquitin-chains for example serve as docking sites for other proteins, which provides important links and relays in cellular signaling cascades (Komander, 2009). The ubiquitin-ligase function of TRAF6 plays an important role in the signal transduction by the receptors of the TIR family or LMP1 (Lamothe et al, 2008; Schultheiss et al, 2001), and TRAF2-induced K63-ubiquitination of cIAPs represents an important switch in non-canonical NF-κB signaling, which will both be described in chapter 1.5.1.

To study the physiological role of TRAFs, several knockout mice have been established that lack individual TRAFs or combinations thereof. Mice deficient in TRAF6, for example, have an osteopetrotic phenotype and are severely hampered in bone and tooth development due to an

impairment in osteoclast function (Lomaga et al, 1999). B cells and fibroblasts derived from these mice are blocked in their responses to IL-1-, CD40- and LPS-stimulation, resulting in impaired proliferation as well as dysfunctional NF-κB and JNK signaling (Lomaga et al, 1999).

Studies with knockout animals lacking the closely related TRAF2 and/or TRAF5 have demonstrated that both molecules have overlapping functions that are redundant in some cases (Au & Yeh, 2006). Disruption of TRAF2 alone leads to premature death of the mice and a high sensitivity of hematopoietic cells to TNFα-induced apoptosis (Yeh et al, 1997). At the same time, TNFα does not induce the JNK pathways in fibroblasts derived from those animals, although the activation of NF-κB is not defective (Lee et al, 1997; Yeh et al, 1997). Likewise, dominant negative TRAF2 blocks CD40-mediated JNK, but not NF-κB signaling, in B cells (Lee et al, 1997). Similarly, B cells devoid of TRAF5 are not impaired in their response to CD40 with regard to JNK and NF-κB activation (Nakano et al, 1999). Studies with fibroblasts obtained from TRAF2/TRAF5 double-deficient animals, however, show that both molecules play redundant roles in TNFα-induced signaling. NF-κB and JNK signaling are both abolished in response to TNFα in TRAF2/5 double-knockout fibroblasts, and these cells are more susceptible to TNFR1-induced apoptosis than TRAF2 single knockout cells (Tada et al, 2001).

1.4 The Death Domain Protein TRADD – Balancing Life and Death in TNFR1 Signaling

Another molecule recruited to LMP1 is TRADD. Similar to TRAFs, TRADD is an adaptor molecule linking receptor activation to cellular signaling pathways (Kieser, 2008; Pobezinskaya & Liu, 2012; Wajant & Scheurich, 2011).

TRADD is a 34 kDa protein and it is an important mediator of signaling cascades induced by death receptors such as TNFR1 or death receptor 3 (DR3) (Hsu et al, 1995; Pobezinskaya et al, 2011; Pobezinskaya & Liu, 2012). Furthermore TRADD was shown to be involved in toll-like receptor (TLR) signaling and in the RIG-like helicase antiviral pathway, and to be a mediator of resistance to TRAIL-induced apoptosis (Cao et al, 2011; Chen et al, 2008; Ermolaeva et al, 2008; Michallet et al, 2008). In most death receptor signaling TRADD plays a dual role. It is important for mediating apoptosis, but also for NF-κB and JNK signaling to promote survival (Hsu et al, 1996b; Hsu et al, 1995; Pobezinskaya et al, 2011). TRADD is a classical adapter molecule that can bind different receptors and downstream proteins to serve as a modulator of protein complex formation. A C-terminal death domain (aa 195 – 305) confers self-association and binding to other proteins with a death domain, such as TNFR1, the Fas-associated death domain protein (FADD) or RIP1 (Hsu et al, 1995). The death domain of TRADD is essential for the induction of signaling from TNFR1, and overexpression of the death domain alone induces

NF-κB signaling as well as apoptosis (Hsu et al, 1995). Additionally, the TRADD N-terminus offers the possibility to bind efficiently to the TRAF-C domain of TRAFs 1, 2 and 3 (Michallet et al, 2008; Park et al, 2000). Upon TNFR1-ligation TRADD is recruited to the receptor via its death domain, and initiates the rapid assembly of the so-called complex I, which consists of TRADD, RIP1, TRAF2 and cIAP1 (Chen & Goeddel, 2002; Ermolaeva et al, 2008; Micheau & Tschopp, 2003; Pobezinskaya et al, 2008). Complex I initiates survival signals via NF-κB. Later a cytosolic complex II forms by replacing TRAF2 and cIAP with FADD and caspase-8, which elicits pro-apoptotic signaling (Hsu et al, 1996b; Micheau & Tschopp, 2003). It is likely that the binding of TRADD to TRAF2 is crucial for tipping the balance between induction of apoptosis or survival by TNFR1. Apoptosis induction by TRADD was greatly enhanced when the TRADD-TRAF2 interaction was reduced by single-mutations of the TRAF-binding residues in the N-terminus of TRADD (Y16A, H65A and S67A) (Park et al, 2000). At the same time sustained NF-κB signaling is responsible for the upregulation of the caspase-8 inhibitor cFLIP, which represses the pro-apoptotic activity of complex II (Kreuz et al, 2001; Micheau et al, 2001). Interestingly, TRADD does not induce apoptosis in the context of LMP1 signaling. Instead, replacing the death domain of TNFR1 with the TRADD binding site of LMP1 renders TNFR1 incapable of inducing programmed cell death (Schneider et al, 2008). The unique role of TRADD in LMP1 signaling will be more extensively described in chapter 1.6.1.

In 2008 four independent reports were published that had undertaken approaches to genetically knock out TRADD. Schneider et al. knocked out TRADD in human DG75 B lymphocytes by homologous recombination, and reported that TRADD is critically involved in the induction of the canonical NF-κB pathway by both TNFR1 and LMP1 (Schneider et al, 2008). A short while later two other groups simultaneously published reports on the generation and study of $TRADD^{-/-}$ mice (Ermolaeva et al, 2008; Pobezinskaya et al, 2008), and a fourth report followed immediately afterwards (Chen et al, 2008).

While the apparent phenotype of TRADD-deficient mice was normal, fibroblasts and BMDMs (bone-marrow derived primary macrophages) obtained from those animals were significantly impaired in their response to TNFα-stimulation with regard to NF-κB and MAPK signaling (Ermolaeva et al, 2008; Pobezinskaya et al, 2008). At the same time TRADD-deficient cells were unable to induce caspase activation in response to TNFR1 stimulation, which shows that TRADD is essential for TNFα-induced apoptosis (Ermolaeva et al, 2008). In line with the importance of TNFR1 signaling in proinflammatory responses, $TRADD^{-/-}$ mice were impaired in their defense against bacterial infection (Ermolaeva et al, 2008). Additionally, TRADD was found to be involved in TRIF-dependent responses to TLR stimulation, and signaling through TLR3,

which depends exclusively on TRIF, was substantially blocked in TRADD-deficient cells (Chen et al, 2008; Ermolaeva et al, 2008; Pobezinskaya et al, 2008).

1.5 Overview of Cellular Signaling Pathways Affected by LMP1

As mentioned in chapter 1.2 LMP1 is capable of engaging and deregulating several cellular signaling pathways involved in transformation, proliferation and cell survival. The following chapter will give an overview of these pathways and how they are regulated by cellular receptors.

1.5.1 Regulation of NF-κB

Deregulated NF-κB signaling is frequently found in tumors and aberrantly growing tissues, and NF-κB generally regulates gene transcription involved in differentiation, survival and proliferation (Karin, 2006). In order to execute such a variety of functions, but still maintain specificity regarding stimulus or cell type, several positive and negative regulatory mechanisms are known that govern the transcriptional activity of NF-κB molecules. In principle, a stimulus leads to a cascade of events including protein phosphorylation, ubiquitination and degradation, which eventually results in the translocation of NF-κB dimers to the nucleus (Hayden & Ghosh, 2008; Schmitz et al, 2004; Vallabhapurapu & Karin, 2009).

The family of NF-κB/Rel transcription factors is composed of five proteins, p65 (RelA), RelB, c-Rel, p50 and p52 (Hayden & Ghosh, 2008; Schmitz et al, 2004). An N-terminal Rel homology domain (RHD) enables these factors to bind to DNA and to form homo- or heterodimers. To prevent transcriptional activity, these NF-κB molecules are usually kept inactive inside the cytoplasm by two major mechanisms: Inhibitor of kappaB (IκB) proteins bind to them and thereby mask the nuclear localization sequence of these molecules, which keeps them inside the cytoplasm. Or they have to be released by active processing of larger protein precursors to release a smaller NF-κB/Rel cleavage product (p100 releases p52 and p105 releases p50) (Vallabhapurapu & Karin, 2009). Two principal pathways are known that regulate the release of distinct sets of NF-κB dimers by different means: the canonical or classical and the non-canonical or alternative NF-κB pathway (figure1-2).

Canonical NF-κB dimers mostly consist of p50 paired with c-Rel or p65, which are maintained in the cytoplasm by IκBα. Following a stimulus, IκBα is rapidly degraded to release the NF-κB heterodimers. This degradation is induced by phosphorylation of the protein at serine residues 32 and 36, followed by its ubiquitination and proteasome-dependent degradation (Chen et al, 1995). Two kinases can specifically phosphorylate IκBα: IκB kinase (IKK) 1 (α) and 2 (β) (Zandi et al, 1997). IKK2 has been shown to be essential to induce phosphorylation of IκBα (Li et al,

1999; Tanaka et al, 1999), although it has more recently been suggested that IKK1 alone can induce canonical NF-κB upon IL-1, but not TNFα stimulation in the absence of IKK2 in IKK2-knockout cells (Solt et al, 2007). Both kinases form the IKK complex together with NEMO (IKKγ), which has an essential regulatory function for canonical NF-κB induction, but plays no role in non-canonical NF-κB signaling (Claudio et al, 2002; Dejardin et al, 2002; Rudolph et al, 2000). The activation of IKK1 and IKK2 in turn depends on the formation of the IKK complex and their phosphorylation by transforming growth factor β-activated kinase-1 (TAK1) or by transautophosphorylation (Delhase et al, 1999; Wang et al, 2001).

Regulatory mechanisms upstream of the IKK complex have most extensively been studied with two receptors, namely the TNFR1 and the Toll/IL-1 (TIL) receptor family. Ligand-activated TNFR1 recruits a complex consisting of TRADD, TRAF2 and RIP. Assembly of this complex is depending on the interaction of the death domains of TNFR, TRADD and RIP1 (Hsu et al, 1996a). TRAF2 homotrimers are recruited to the N-terminal TRAF-binding domain of the receptor-bound TRADD-proteins, and subsequently tether cellular inhibitors of apoptosis (cIAPs) 1 and 2 to the receptor complex (Hsu et al, 1996b; Zheng et al, 2010). TRAF2 and cIAPs cooperate in K63-ubiquitination of RIP1, a process in which the E3-ligase function of cIAPs seems to be essential (Bertrand et al, 2008). Ubiquitinated RIP1 proteins serve as an adapter platform that can bind NEMO to recruit the IKK complex, or for the binding of the TAB/TAK complex (Ea et al, 2006; Kelliher et al, 1998; Zhang et al, 2000). Additionally TRAF2 stabilizes the IKK complex by binding the leucin-zipper motives of IKK1 and 2 through the TRAF2 RING domain (Devin et al, 2001). As mentioned before in chapter 1.3, the role of TRAF2 can be replaced by TRAF5, and cells lacking either TRAF2 or TRAF5 are still functional in inducing NF-κB, while TRAF2/TRAF5 double knockout cells are not (Nakano et al, 1999; Tada et al, 2001; Yeh et al, 1997).

The activation of NF-κB by Toll/IL-1 receptors (TIR), however, is critically dependent on TRAF6 (Cao et al, 1996; Lomaga et al, 1999). Upon receptor ligation, the adapter protein MyD88 binds to the receptor and mediates recruitment of IL-1R-associated kinase 1 (IRAK-1) and 4 and TRAF6 (Qian et al, 2001; Verstrepen et al, 2008; Wesche et al, 1997). Subsequently, IRAK-4 becomes activated by autophosphorylation, which leads to phosphorylation and activation of IRAK-1 (Cheng et al, 2007; Kollewe et al, 2004; Verstrepen et al, 2008). The ubiquitin-ligase function of the RING domain of TRAF6 leads to K63-autoubiquitination of TRAF6, but also to the K63-ubiquitination of IRAK-1 and -4 (Conze et al, 2008). This recruits a complex consistent of TAB1, TAB2 and TAB3 as well as the MAP3K TAK1, which then activates the IKK complex (Ishitani et al, 2003; Qian et al, 2001; Takaesu et al, 2000; Wang et al, 2001).

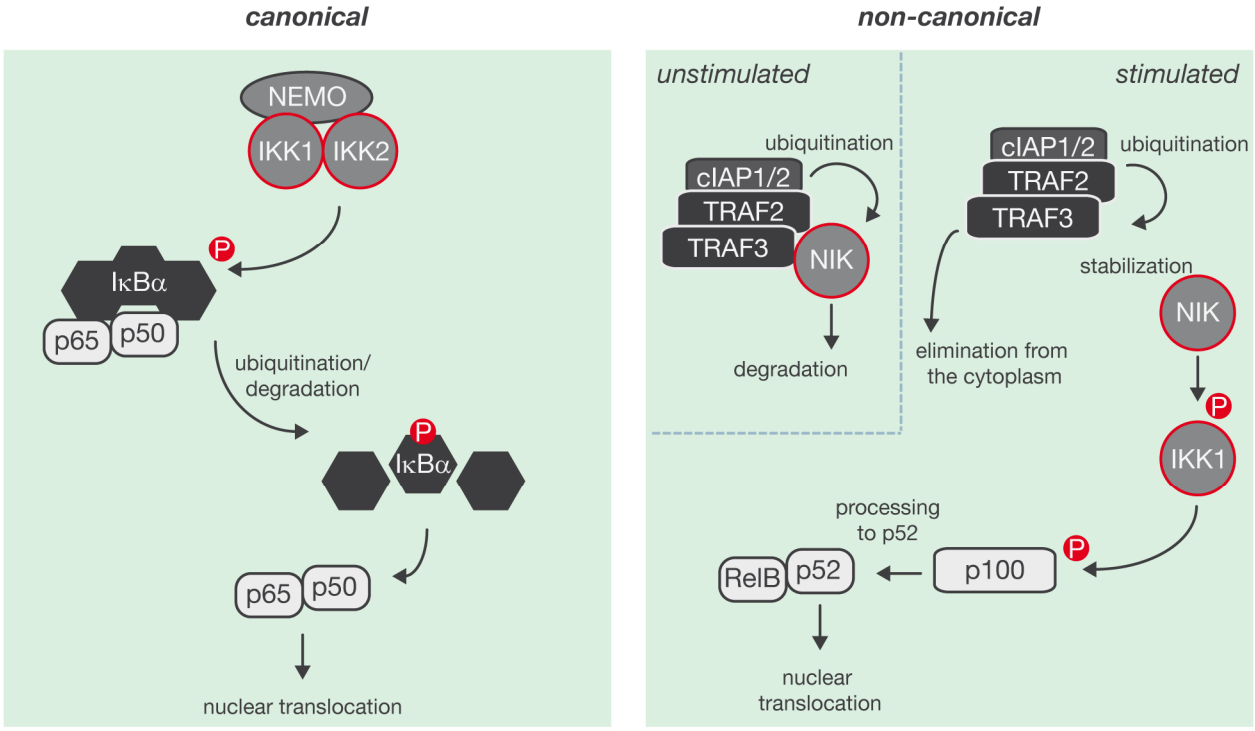

Figure 1-2. Principles of NF-κB signaling. For canonical signaling the IKK complex is activated, which leads to the phosphorylation of IκB. Subsequently, IκB is degraded by the proteasome and NF-κB heterodimers are released to translocate to the nucleus. The non-canonical NF-κB pathway is inactive as long as the kinase NIK is complexed with TRAF2/3, which leads to the TRAF2-dependent ubiquitination and subsequent degradation of NIK. Upon ligation of the receptor, TRAF3 now becomes the substrate for ubiquitination and NIK is released and stabilizes to phosphorylate IKK1. IKK1 in turn phosphorylates the NF-κB precursor p100, which is then processed to p52. p52 dimerizes with RelB to translocate to the nucleus.

TRAF proteins are also of great importance for the regulation of the non-canonical NF-κB pathway (figure 1-2). Here, however, they initially inhibit the activation of the pathway instead of propagating it. In unstimulated cells TRAF2 and TRAF3 form a cytoplasmic complex that binds the ubiquitously expressed NF-κB inducing kinase (NIK) and continuously promotes its degradation (Liao et al, 2004; Zarnegar et al, 2008). This is mediated by TRAF2-dependent recruitment of cIAPs, which facilitate K48-ubiquitination and degradation of NIK (Vallabhapurapu et al, 2008; Zarnegar et al, 2008). Upon activation of a receptor cIAPs are K63-ubiquitinated by TRAF2, prompting the substrate for K48-ubiquitination to change from NIK to TRAF3 (Liao et al, 2004; Sanjo et al, 2010; Vallabhapurapu & Karin, 2009; Vallabhapurapu et al, 2008). This leads to the degradation of TRAF3 and the stabilization and gradual enrichment of NIK (Liao et al, 2004; Qing et al, 2005). NIK is now able to phosphorylate and activate IKK1, which in turn phosphorylates the NF-κB precursor p100 (Xiao et al, 2004; Xiao et al, 2001). p100 is processed to p52, which complexes with RelB and translocates to the nucleus to initiate gene transcription

(Xiao et al, 2001). Notably, TRAF2 is needed for both activation and inhibition of the non-canonical NF-κB pathway. Knockout of TRAF2 leads to hyperactivation of the non-canonical NF-κB pathway due to continuous processing of p100, which shows that TRAF2 is needed for the inhibition of the pathway (Grech et al, 2004; Vallabhapurapu et al, 2008; Zhang et al, 2009). Even so, p100-processing and p52-translocation are not induced upon CD40 activation, if the association of TRAF2 with CD40 is blocked by mutation of the TRAF2-binding site of the receptor, which demonstrates that TRAF2 is also essential for the tethering of the TRAF/NIK-complex to the receptor and thereby for the activation of the pathway (Hauer et al, 2005). The inhibitory role of TRAF3 for the non-canonical NF-κB pathway is also supported by the fact that TRAF3-deficient cells constitutively induce the processing of p100 to p52 (He et al, 2006; Song & Kang, 2010; Vallabhapurapu et al, 2008).

1.5.2 MAPK Pathways

In general, mitogen-activated protein kinase (MAPK) signaling cascades are involved in a wide array of cellular responses such as mitogen, cytokine and stress responses, and they can regulate a suiting amount of cellular fates including survival, apoptosis, proliferation and differentiation (Cargnello & Roux, 2011; Raman et al, 2007; Zhang & Liu, 2002). MAP-kinases are activated by phosphorylation of specific conserved serine/threonine and tyrosine residues inside the kinase domain, and the mode of signal transduction follows a sequence of hierarchical phosphorylation events (figure 1-3) (Cargnello & Roux, 2011). The MAPK, which are the classical effector MAP-kinases including ERK1/2, JNK and p38, phosphorylate and thereby activate specific transcription factors. JNK activates transcription factors of the AP-1 family, ERK can phosphorylate numerous substrates including c-myc, STAT3 and CREB, and p38 is involved in regulation of Hsp27 and transcription factors like ATF1 or STAT1, among others (Cuadrado & Nebreda, 2010; Weston & Davis, 2007; Yoon & Seger, 2006). The phosphorylation cascade includes three levels of MAP-kinases in a way that activated MAP3K (like Raf or TAK1) phosphorylate the respective downstream MAP2K (like MEK or MKKs), which in turn phosphorylate their suitable MAPK (Cargnello & Roux, 2011; Raman et al, 2007; Weston & Davis, 2007; Yoon & Seger, 2006). A general overview of MAPK signaling cascades is depicted in figure 1-3. The MAP3Ks are regulated by different mechanisms originating at varying receptor signaling complexes. The ERK cascade for example can be activated by small GTPases (like Ras), which in turn are activated through signaling events mediated by the ligation of the respective receptor (Raman et al, 2007; Yoon & Seger, 2006). TRAF proteins can also play an important role in the activation of the MAPK pathways. Their involvement in MAPK activation is quite similar to the part they play in the activation of the canonical NF-κB pathway (chapter 1.5.1). TRAF2, for example, is a critical mediator for the activation of JNK in response to TNF or

CD40 (Hostager et al, 2003; Lee et al, 1997). It can recruit numerous MAP-kinases such as ASK1 (apoptosis signal-regulating kinase) or GCK (germinal center kinase) to induce MAPK pathways and AP-1 activation (Hoeflich et al, 1999; Shi et al, 1999; Yuasa et al, 1998).

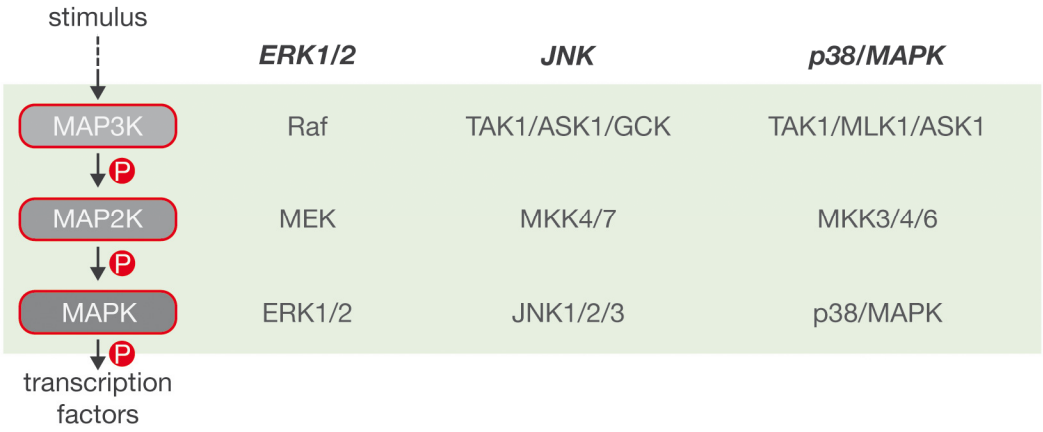

Figure 1-3. Overview of common MAPK signaling cascades. Upon an external stimulus the MAP3K are activated either by small GTPases or by a specific signaling complex. Subsequently, MAP3K phosphorylate MAP2K, which in turn phosphorylate MAPK. These finally activate specific transcription factors.

1.5.3 Regulation of PI3K/Akt

One of the major pathways involved in growth stimulation, proliferation, cell cycle regulation and the rescue from apoptosis is the PI3K/Akt pathway. Once activated, the central kinase Akt phosphorylates and thereby regulates a wide array of substrates ranging from pro-apoptotic factor BAD over GSK-3 to IKKs (Franke, 2008; Ozes et al, 1999). Akt is often found deregulated or hyperactivated in tumors and it is linked to a bad prognosis of cancer patients (Carnero, 2010; LoPiccolo et al, 2008). The PI3K/Akt signaling cascade is triggered by the binding of the regulatory p85 subunit of PI3K to phosphorylated tyrosine residues of activated receptors (e.g. IGF-R) via its SH2-domain[5]. Subsequently, p85 is tyrosine-phosphorylated by Src family kinases, which represses its inhibitory effect on the catalytic subunit p110 (Cuevas et al, 2001). This releases p110 and enables it to phosphorylate phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P) to the second messengers phosphatidylinositol-3,4-bisphosphate (PI-3,4-P) and phosphatidylinositol-3,4,5-trisphosphate (PIP_3), respectively (Auger et al, 1989; Ruderman et al, 1990; Yu et al, 1998). PIP_3 now recruits the downstream kinases Akt and PDK1 via their PH-domains to the membrane and bring them

[5] SH2-domain, (Src-homology 2 domain) protein structure that facilitates binding to phosphorylated tyrosine residues

into proximity (Andjelkovic et al, 1997). This triggers the phosphorylation of Akt by PDK1 at Thr308 (Alessi et al, 1997; Ding et al, 2010), which is enhanced by previous phosphorylation at Ser473 by mTORC2 (Sarbassov et al, 2005; Scheid et al, 2002). The full activity of Akt depends on the phosphorylation of both residues (Alessi et al, 1996). Negative regulation of the PI3K/Akt pathway includes the proteasome-dependent degradation of Akt, dephosphorylation of PIP_3 by PTEN or dephosphorylation of Akt by the phosphatases PHLPP or PP2A (Brognard et al, 2007; Gao et al, 2005; Maehama et al, 2001; Millward et al, 1999). A schematic overview of the regulation of the PI3K/Akt pathway is depicted in figure 1-4.

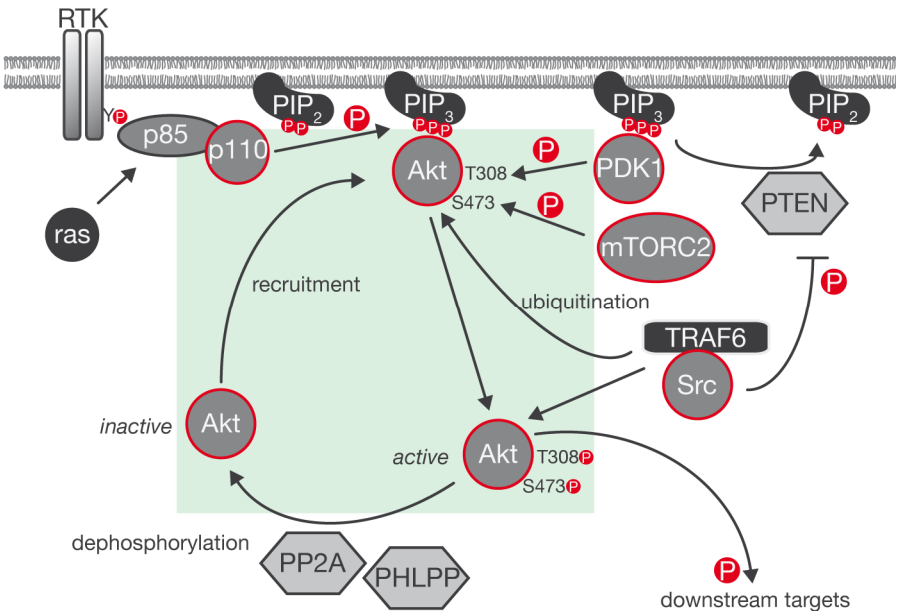

Figure 1-4. Schematic overview of the regulation of Akt. Ligation of a receptor tyrosine kinase (RTK) leads to tyrosine-phosphorylation within its cytoplasmic tail. PI3K recruits to these phospho-tyrosines and catalyzes the conversion of PIP_2 to PIP_3. PIP_3 brings PDK1 and Akt into proximity resulting in the phosphorylation of Akt at Thr308. mTORC2 activates Akt by phosphorylating the Ser473 residue. Activated Akt re-translocates to the cytoplasm to phosphorylate several downstream targets involved in cell cycle regulation, cell growth and survival. The phosphatases PTEN and PP2A or PHLPP terminate the signal by dephosphorylating PIP_3 and Akt, respectively. TRAF6 aids Akt-recruitment and activation by ubiquitination of Akt, and by complexing with c-Src.

There have also been reports that TRAF6 can play an important role in the activation of Akt, which eventually can lead to actin filament remodeling (Vandermoere et al, 2007; Wang et al, 2006). Interaction of TRAF6 and c-Src, which occurs via a unique polyproline motif within the TRAF-C domain of TRAF6, was shown to be essential for Akt activation by TRAF6 (Wong et al, 1999). Both overexpression of TRAF6 or stimulation with IL-1 similarly induce the PI3K/Akt

pathway (Wang et al, 2006). A more recent study further demonstrated that the E3 ubiquitin-ligase function of TRAF6 plays an important role in the activation of Akt. TRAF6-dependent ubiquitination of Akt was shown to enhance its recruitment to the membrane upon growth factor stimulation (Yang et al, 2009b). Furthermore, especially phosphorylation at Thr308 was reduced in TRAF6$^{-/-}$ MEFs upon IGF-1 stimulation, and overexpression of TRAF6 in turn led to pronounced Akt phosphorylation at Thr308 (Yang et al, 2009b). It seems that TRAF6 can be involved in PI3K/Akt activation by different mechanisms, which might vary depending on the stimulus.

IRAK-1, which is an important co-factor of TRAF6 in TIR-dependent signaling, was also shown to be involved in the activation of Akt by IL-1β (Neumann et al, 2002). Several reports further demonstrated that Ras plays an important role in the activation of PI3K, and that this could also be mediated by TRAF6 (Kodaki et al, 1994; Rodriguez-Viciana et al, 1994; Wang et al, 2006). Furthermore, it was suggested that TRAF6 and c-Src dependent activation of Akt in response to IL-1 can lead to AP-1 activation (Funakoshi-Tago et al, 2003). Src itself was also implicated in the positive regulation of Akt by phosphorylating and thereby inhibiting PTEN (Lu et al, 2003).

1.5.4 JAK/STAT Signaling Pathways

Aberrant activation of signal transducers and activators of transcription (STAT) is often found in tumors (Inghirami et al, 2005), and elevated levels of activated STAT proteins have been reported to be associated with EBV and LMP1 in NPC (Hannigan et al, 2011; Liu et al, 2008; Ma et al, 2008). Generally, STAT transcription factors play a central role in conveying signaling responses to cytokine stimulation and are involved in gene regulation associated with survival, proliferation and differentiation. So far, seven members of the STAT family have been identified in mammals. They are activated either by direct phosphorylation through growth factor receptor associated kinases, or canonically by Janus kinases (JAK). The principle activation of the JAK/STAT pathway is best exemplified with cytokine receptors like the gp130 family of receptors. These receptors consist of a preformed dimer, which remains inactive due to a specific conformational arrangement (Livnah et al, 1999; Remy et al, 1999). The cytosolic tails of the receptors are constantly associated with JAK proteins, which bind to a membrane-proximal region containing so called box1 and box2 motifs[6] (Giese et al, 2003; Haan et al, 2002; Haan et al, 2000; Murakami et al, 1991; Usacheva et al, 2002). Upon receptor ligation, conformational changes of the receptor lead to the trans-autophosphorylation of JAKs and the subsequent phosphorylation of tyrosine residues within the far C-termini of the receptors (Remy et al, 1999). These phospho-tyrosines serve as anchors for the SH2-domains of inactive STAT proteins,

[6] box1/box2 motifs, highly conserved motifs in the signaling regions of different receptors (e.g. cytokine receptors) that can bind and activate JAKs. Box 1 consists of a distinct set of proline residue (PxxPxP).

which are transiently recruited to the receptor (Greenlund et al, 1994; Hemmann et al, 1996). This recruitment is followed by STAT phosphorylation on a specific tyrosine residue (Tyr705 in STAT3) by JAKs (Kaptein et al, 1996). Phosphorylated STAT is released from the receptors to form dimers with the help of the same SH2-domains needed for receptor binding. These activated dimers translocate to the nucleus, where they drive transcription of target proteins. A second phosphorylation step at Ser727, which was shown to be catalyzed by MAPK pathways, is discussed to have modulatory effects on the transcriptional activities of STAT proteins, depending on the cell type and stimulus (Andres et al, 2013; Goh et al, 1999; Kovarik et al, 2001; O'Rourke & Shepherd, 2002).

STAT proteins are involved in several distinctive cellular functions and are activated by specific stimuli. STAT1 activity, for example, is induced by Interferon signaling, while the key activators of STAT3 are growth factors and inflammatory cytokines of the IL-6 family (Pensa et al, 2009; Schindler et al, 2007). The biological functions of both molecules differ greatly and can take opposing roles in tumorigenesis (Pensa et al, 2009). Generally, STAT1 is considered to be a tumor suppressor. For example, STAT1 can inhibit metastatic growth and angiogenesis by interfering with the expression of MMPs or the activity of VEGF, respectively (Battle et al, 2006; Huang et al, 2002). Furthermore, STAT1 is involved in cell cycle arrest by promoting the expression of specific CDK (cyclin-dependent kinase) inhibitors (Chin et al, 1996; Dimberg et al, 2003). Several pro-apoptotic functions, like caspase activation, p53 co-regulation or upregulation of Fas and Fas ligand, are also associated with Interferon-dependent STAT1 activity (Pensa et al, 2009). In contrast, STAT3 has commonly been described to be an oncogene that is frequently found to be constitutively active in numerous tumors (Aggarwal et al, 2009; Fagard et al, 2013; Pensa et al, 2009). STAT3 can promote growth and cell cycle progression through upregulation of cyclin D1 or myc (Kiuchi et al, 1999; Masuda et al, 2002), and it mediates the expression of anti-apoptotic proteins of the Bcl-2 family (Liu et al, 2003; Zushi et al, 1998). Apart from that, STAT3 supports metastasis and angiogenesis by targeting genes encoding for members of the MMP family, like MMP-9 (Song et al, 2008), or VEGF (Niu et al, 2002; Wei et al, 2003).

1.6 Signaling Properties of LMP1

Apart from its ligand-independent, constitutive activation, the signaling properties of LMP1 are strikingly reminiscent of the tumor necrosis factor receptor (TNFR) family and especially of the B cell activating receptor CD40, (Kilger et al, 1998; Uchida et al, 1999). For example, both molecules recruit TRAFs to trigger a similar lineup of signaling pathways, and both localize to lipid rafts for signal induction (Kaykas et al, 2001). In fact, a fusion protein between the

extracellular domain of CD40 and the signaling domain of LMP1 completely mimics the functions of CD40 in transgenic mice and rescue the phenotype of a CD40 knockout (Rastelli et al, 2008). However, the constitutively active nature of LMP1 seems to render it more efficient for signal transduction than an inducible receptor like CD40 (Kaykas et al, 2001). Furthermore, LMP1 and CD40 do not share any significant sequence homology apart from the common TRAF-binding sites, indicating that both molecules evolved independently, and their molecular mechanisms are not fully equivalent (Graham et al, 2010). The following subchapters will deal with the major signaling pathways that are induced by LMP1, and with what is known so far about the different regulatory mechanisms, which LMP1 is capable of executing in order to trigger these pathways.

Apart from the pathways described in the following sections, LMP1 is also able to induce the activation of IRF7 (Ersing et al, 2013). However, IRF7 has not been addressed in the course of this work, so the mechanisms leading to IRF7 activation by LMP1 will not be described in detail.

1.6.1 Induction of the NF-κB- and JNK-Pathways by LMP1 – a Paradigm for the Roles of TRAFs and TRADD?

Similar to the receptors of the TNFR family, LMP1 does not display intrinsic enzymatic activity and relies on adapter proteins to build up signaling complexes that catalytically trigger signaling. To do so, and again similar to TNF-receptors, LMP1 engages proteins of the TRAF and TRADD family. However, despite the similarities, the mechanisms of signal transduction by LMP1 differ from TNF-receptors like CD40 in several aspects. This chapter will discuss the current view of how LMP1 activates the JNK and the NF-κB pathways (figure 1-5).

LMP1 can engage both the canonical and the non-canonical NF-κB pathway, and it was shown that NF-κB-activity is essential for LMP1-induced cell transformation (Cahir McFarland et al, 1999; Eliopoulos et al, 2003a; He et al, 2000; Paine et al, 1995). Both CTAR1 and CTAR2 have been shown to regulate NF-κB induction, although CTAR2 seems to contribute to over 70% of total NF-κB activity detected by an NF-κB reporter construct, while CTAR1 is responsible for only 30% (Huen et al, 1995; Mitchell & Sugden, 1995). NF-κB signaling initiated at CTAR1 was described to be essential for the primary initiation of transformation, while CTAR2-dependent NF-κB signals have been attributed to the long-term outgrowth of LMP1-transformed cells (Kaye et al, 1995). The elucidation of the exact mechanisms by which LMP1 triggers NF-κB has been the research topic for many years, and although extensive advances have been made in the field, some aspects still remain unresolved and controversial.

The Non-Canonical NF-κB Pathway Induced by LMP1

It is generally accepted that the TRAF-binding site P_{204}xQxT in CTAR1 is responsible for the NEMO-independent induction of the non-canonical NF-κB pathway, whereas mutation of CTAR2

does not affect p100 to p52 processing (Atkinson et al, 2003; Eliopoulos et al, 2003a; Luftig et al, 2004; Saito et al, 2003). Furthermore it was shown that LMP1-induced p100-processing does not require TRAF6 and depends on both NIK and IKK1 (Luftig et al, 2004; Song & Kang, 2010). This suggests that LMP1 induces NIK-stabilization through the “classical” mechanism by binding the TRAF2/3-cIAP complex to induce TRAF3 degradation (see chapter 1.5.1). This is supported by the fact that overexpression of TRAF3 reduces CTAR1-dependent NF-κB activation (Devergne et al, 1996). Even so, studies with inducible LMP1 in B cells have shown that, in contrast to CD40, stimulation of LMP1 did not provoke TRAF3 degradation (Brown et al, 2001). Therefore LMP1 must be capable of inducing the non-canonical NF-κB pathway through different means than cellular receptors. Nonetheless, TRAF3 must play an inhibitory role in LMP1-induced non-canonical NF-κB activation, because overexpression of TRAF3 significantly reduced LMP1-induced processing of p100 (Song & Kang, 2010).

The role of TRAF2 in LMP1-dependent activation of the non-canonical NF-κB pathway has not been clearly established. This is in part due to the fact that depletion of TRAF2 leads to the constitutive activation of p100-processing independently of LMP1, as described before (chapter 1.5.1 and Song & Kang, 2010). However, the expression of dominant negative TRAF2 lacking the RING domain significantly reduced CTAR1-induced NF-κB, which suggests a critical role for TRAF2 in this signaling pathway (Kaye et al, 1996). Similarly, overexpression of TRAF2 blocked the processing of p100 to p52 in LMP1-expressing cells, which shows that TRAF2 plays an inhibitory role for this pathway in the context of LMP1 signaling (Song & Kang, 2010). Furthermore, TRAF6 might also have an impact on NF-κB signaling by CTAR1. Expression of a dominant negative TRAF6 mutant lacking the RING-domain greatly reduced CTAR1-induced NF-κB activity (Schultheiss et al, 2001), although the underlying mechanism is not clear. Additionally, CTAR1 has been shown to regulate the activation of unusual, alternative forms of NF-κB. Homodimers of p50 in complex with Bcl-3 were shown to mediate CTAR1-dependent upregulation of EGFR (Thornburg & Raab-Traub, 2007), and it has been reported that CTAR1 can induce the formation of alternative p65/p52 heterodimers (Song & Kang, 2010).

LMP1-Dependent Canonical NF-κB Signaling

Activation of the canonical NF-κB pathway has been largely associated with CTAR2, and the sequence P_{379}VQLSY was shown to be essential for NF-κB activation (Floettmann & Rowe, 1997). Inhibition of IKK2 or lack of NEMO leads to the complete block of CTAR2-induced NF-κB activation (Boehm et al, 2010). NEMO, however, can seemingly be utilized by LMP1 in specific ways that differ from TNFR1 or CD40. Jurkat cells with mutated NEMO, which lacks either the Zn-finger domain or in which a part of the coiled coil region is deleted, still induced NF-κB upon LMP1 expression, but not upon CD40 or TNFR ligation (Boehm et al, 2010). Utilization of

siRNAs furthermore suggested that the roles of IKK1 and IKK2 in LMP1-dependent canonical NF-κB activation can be partially redundant (Gewurz et al, 2012).

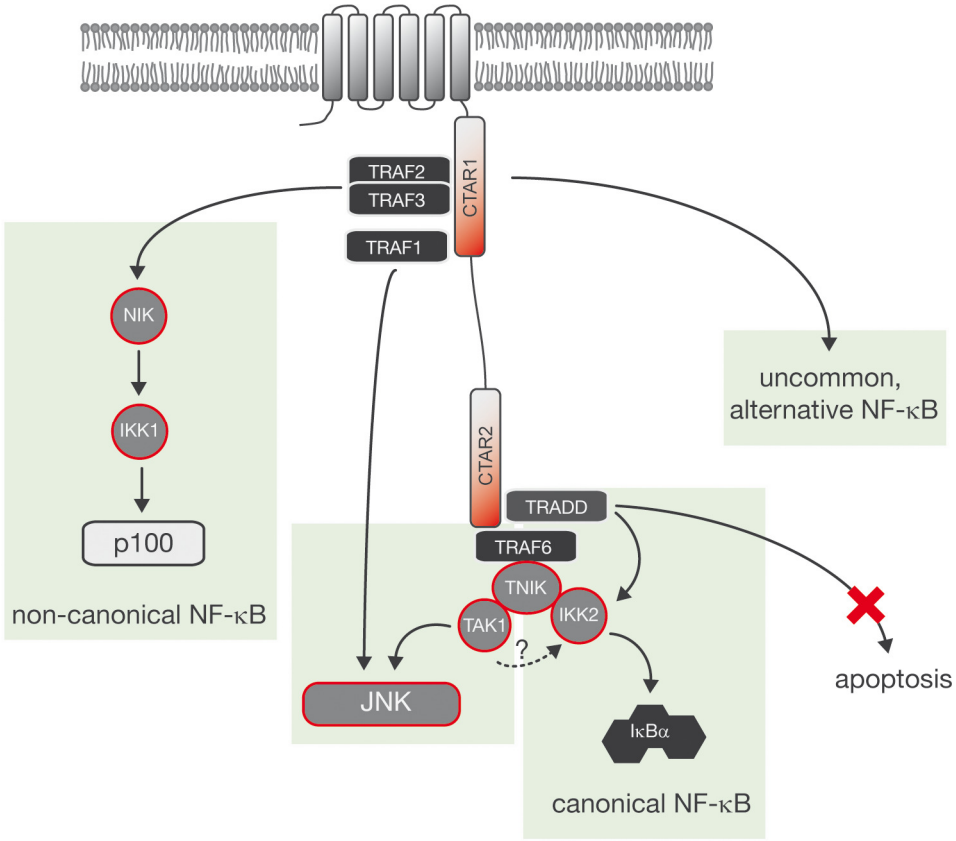

Figure 1-5. Schematic overview of LMP1-induced JNK and NF-κB pathways. Activation of the non-canonical NF-B pathway by LMP1 is induced through CTAR1-dependent activation of NIK and IKK1. TRAF3 is a negative regulator of this pathway, but is not degraded upon signal-initiation by LMP1. CTAR1 was additionally shown to induce uncommon forms of NF-κB like dimers of p50 and Bcl-6. The canonical NF-κB and JNK pathways originate at CTAR2, and TRAF6 is a critical mediator by recruiting TNIK. TNIK recruits TAK1 and IKK2, which leads to the bifurcation of TAK1-dependent JNK and IKK2-dependent NF-κB signaling. TRADD plays a role in the activation of canonical NF-κB by facilitating the recruitment of IKK2 to the complex. Importantly, TRADD-recruitment to LMP1 does not lead to apoptosis induction.

Early studies demonstrated that CTAR2-dependent activation of NF-κB can be reduced by the expression of dominant negative TRAF2, which lacks the RING-domain (Kaye et al, 1996). However, B cells lacking TRAF2 are not compromised in their ability to induce degradation of IκBα upon ligation of a chimeric CD40-LMP1 receptor (Xie & Bishop, 2004). Yet, the function of TRAF2 in NF-κB activation may have been substituted by TRAF5, as described before (chapter 1.5.1 and Nakano et al, 1999; Tada et al, 2001; Yeh et al, 1997). Nonetheless, MEFs deficient in

both TRAF2 and TRAF5 induced NF-κB almost as well as wildtype MEFs and did not fail in CTAR2-dependent IKK2 activation, suggesting that these two proteins are dispensable for LMP1-dependent canonical NF-κB activation (Luftig et al, 2003; Wu et al, 2006).

In contrast, CTAR2-mediated NF-κB signaling depends critically on TRAF6. Expression of dominant negative TRAF6 lacking the RING domain greatly reduced LMP1-induced NF-κB activation (Schultheiss et al, 2001). Similarly, the lack of TRAF6 in MEFs significantly diminished the ability of LMP1 to induce nuclear translocation of RelA or activation of NF-κB-dependent transcription in comparison to wildtype cells (Luftig et al, 2003). Additionally, isolated CTAR2 triggered activation of NF-κB dependent on TRAF6 (Schneider et al, 2008). The role of TAK1 in NF-κB activation by LMP1 has been discussed controversially. siRNA-mediated knockdown of TAK1 in 293T cells led to a reduction of CTAR2-induced IKK2 activity (Wu et al, 2006). Inhibition of TAK1 by a chemical inhibitor, however, did not significantly reduce LMP1-induced NF-κB activity (Uemura et al, 2006). This discrepancy could be due to the different challenges applied to block TAK1 activity, and it is possible that inhibition of the kinase activity of TAK1 was insufficient to reduce the pathway activation. Apparently, another important mediator of TIR-induced NF-κB signaling plays a role in the signal transduction by LMP1. 293 cells lacking IRAK-1 were greatly compromised in their ability to induce NF-κB after LMP1 expression compared to control cells (Luftig et al, 2003). However, lack of IRAK-1 does not interfere with LMP1-induced IKK activation or NF-κB translocation (Song et al, 2006). The kinase activity of IRAK-1 seems to be expendable as well, since mutation of an essential residue K239 in the kinase domain of IRAK-1 only diminished the ability of LMP1 to induce NF-κB (Song et al, 2006). Nevertheless, phosphorylation of p65 at S536 was abolished in cells lacking IRAK-1, even though this was unaffected by mutation of the kinase domain, suggesting that IRAK-1 plays a more downstream role in the activation of canonical NF-κB by LMP1 (Song et al, 2006).

The death domain protein TRADD was also identified to be an important mediator for CTAR2 signaling, and yeast-two-hybrid studies indicated that it is a direct interaction partner of the Y_{384}YD motif at the far C-terminal end of LMP1 (Izumi & Kieff, 1997). In contrast to TNFR1 signaling, however, TRADD does not exert pro-apoptotic signaling in association with LMP1 (Izumi et al, 1999b; Izumi & Kieff, 1997; Kieser et al, 1999; Schneider et al, 2008). Instead, overexpression of TRADD and LMP1 together leads to enhanced activation of both NF-κB and JNK (Eliopoulos et al, 1999a; Izumi & Kieff, 1997). It thus seems that LMP1 utilizes TRADD in a fashion that differs from TNF receptors, which might explain its inability to induce apoptosis in LMP1 signaling. It was shown that TRADD is recruited to LMP1 in the absence of its death domain and the death domain alone does not associate with LMP1 (Kieser et al, 1999). At the same time LMP1 does not feature a death domain, but can still recruit TRADD. This supports the

fact that TRADD is recruited to LMP1 in a unique way via its N-terminal domain. If the CTAR2 domain of LMP1 is fused to the extracellular domain of TNFR1, TNFα stimulation results in recruitment of TRADD and activation of NF-κB, but not in the induction of apoptosis (Schneider et al, 2008). It is not entirely clear in which way the death domain of TRADD is important for the initiation of signaling by LMP1. Overexpression of a dominant negative TRADD, which lacks the entire death domain, interferes with LMP1-induced NF-κB and p38/MAPK signaling, but does not block the JNK pathway (Kieser et al, 1999; Schultheiss et al, 2001). However, after crippling of the death domain in a way that abolishes TNFR1- or self-association but still allows some death domain interaction (Hsu et al, 1995; Park & Baichwal, 1996) TRADD still enhances NF-κB in concert with LMP1 (Izumi et al, 1999b).

It was shown that in B cells TRADD mediates the recruitment of IKK2 to the LMP1 signaling complex. LMP1-induced IKK2 activity was blocked in TRADD$^{-/-}$ cells and no IKK2 could be co-precipitated with LMP1 in the absence of TRADD (Schneider et al, 2008). Even so, knockdown of TRADD in 293 cells via siRNA did not significantly affect LMP1-induced NF-κB activity, although it is possible that residual amounts of TRADD could be responsible for this (Wu et al, 2006).

Despite its usual inhibitory function, TRAF3 seems to play an essential role in the activation of the canonical NF-κB pathway by LMP1 in mouse B cell lines. Stimulation of LMP1 in A20.2J B cells lacking TRAF3 did not lead to the degradation of IκBα, in contrast to CD40 stimulation in these cells (Xie et al, 2004). The same was true for the activation of JNK and p38, which were both defective in TRAF3$^{-/-}$ B cells regarding LMP1 activation, but not CD40 stimulation (Xie et al, 2004). This means that LMP1 and CD40 utilize TRAF3 in distinctive ways, and TRAF3 may play a much more important role in LMP1 signaling in certain cell types than in signaling by TNF receptors.

Induction of the JNK Pathway by LMP1

The P_{379}VQLSY TRADD binding sequence within CTAR2, and at its core the tyrosine residue at position 384, is also the major inducer of JNK activation by LMP1, which can be blocked by dominant negative SEK1 (Eliopoulos & Young, 1998; Kieser et al, 1999; Kieser et al, 1997). However, dominant negative TRADD lacking the death domain did not reduce the ability of LMP1 to induce JNK signaling, suggesting that TRADD is not critically involved in this pathway (Kieser et al, 1999). Supporting this, studies conducted with RNAi demonstrated that no negative effect on the initiation of JNK signaling could be achieved by the lack of TRAF2, RIP or TRADD, (Wan et al, 2004), although one study showed that overexpression of TRADD enhanced JNK signaling, and expression of dominant negative TRAF2 could reduce LMP1-induced JNK activity (Eliopoulos et al, 1999a). Even so, the lack of TRADD in human or TRAF2 in mouse B cells did

not interfere with the JNK pathway induced by LMP1, clearly indicating that neither molecule is essentially needed for the activation of JNK by LMP1 in B cells (Schneider et al, 2008; Xie & Bishop, 2004).

By using knockout cells it was shown that TRAF6 and TAK1, but not TAB2, are essential for JNK activation by LMP1 (Wan et al, 2004). Also, inhibition of TAK1 greatly reduced LMP1-induced JNK phosphorylation in 293 cells (Uemura et al, 2006). This would suggest that JNK activation by LMP1 is less reminiscent of TNFR1 than TIR. However, also RNAi against mediators of TIR-induced signaling MyD88, IRAK-1 and IRAK-4 had no effect on JNK activation (Wan et al, 2004). Despite the critical involvement of TRAF6 in JNK signaling, overexpression of a dominant negative TRAF6 with a mutation of the RING-domain, which blocked p38/MAPK activation and reduced the induction of NF-κB, had no effect on the JNK pathway (Schultheiss et al, 2001). This indicates that the E3-ubquitin ligase function of TRAF6 might not be critically needed for the induction of the JNK pathway by LMP1.

As for the importance of JNK signaling for LMP1-induced transformation, it was shown that inhibition of JNK by the inhibitor SP600125 or expression of dominant negative JNK blocked proliferation of LMP1-transformed Rat-1 cells and LCLs (Kutz et al, 2008). In line with this, SP600125 significantly delayed tumor formation in SCID mice xenotransplanted with LCLs (Kutz et al, 2008).

Only very few studies so far have reported on the possibility of CTAR1 being responsible for some JNK signaling as well. In Rat-1 cells, mutation either of CTAR1 or CTAR2 were shown to reduce JNK1 activity to approximately 50 % (Kutz et al, 2008). TRAF1 can be a mediator of JNK activation at CTAR1. Expression of LMP1 with a deletion of P_{379}VQLSY in the EBV-negative Burkitt cell line BJAB still induced JNK activation depending on CTAR1. It was shown that the very strong expression of TRAF1 in these cells is responsible for this, and overexpression of TRAF1 was able to induce JNK signaling from CTAR1 in 293 cells (Eliopoulos et al, 2003b). Similarly, signal-induction by a chimeric receptor consisting of the extracellular CD40 domain and the intracellular LMP1 signaling domain failed to activate JNK when either CTAR1 or CTAR2 were mutated (Busch & Bishop, 2001; Xie & Bishop, 2004).

The seemingly conflicting results concerning JNK activation by CTAR1 and CTAR2 again demonstrate that the mechanisms of LMP1 signaling can vary depending on the cell type and physiology or intracellular environment of the cell. Interestingly, simultaneous expression and activation of two independent inducible CD40-LMP1 receptors that lack either the CTAR1 or the CTAR2 domain restores JNK activation upon ligation (Xie & Bishop, 2004). This means that

CTAR1 and 2 can cooperate to induce cellular signaling pathways and that both domains need to be physically present in order to trigger signaling in certain situations.

Bifurcation of JNK and Canonical NF-κB Activation

CTAR2-dependent activation of both NF-κB and JNK depends on the exact same core sequence P379 - Y384 (Floettmann & Rowe, 1997; Kieser et al, 1999). This and the fact that the activation of JNK and canonical NF-κB share the important signaling protein TRAF6 imply that the induction of these two pathways must originate at the same receptor-proximal signaling complex, and bifurcate further downstream. In fact, Shkoda et al. showed that the germinal center kinase TNIK (TRAF2- and Nck-interacting kinase) serves as an essential mediator of LMP1-induced canonical NF-κB and JNK signaling, and that the induction of both pathways diverges at the level of TNIK (Shkoda et al, 2012). Deletion of the N-terminal kinase domain of TNIK completely blocked the induction of canonical NF-κB by the kinase, but no reduction of JNK activity was observed when the kinase domain was mutated. Instead, expression of the kinase domain alone was sufficient to induce canonical NF-κB, whereas JNK was induced by expression of the C-terminal GCKH (germinal center kinase homology) domain (Shkoda et al, 2012). This is supported by the fact that TNIK forms a complex with TAK1 and TAB2, and that TAK1, which is important for LMP1-dependent JNK activation, as discussed before, associates with the GCKH domain of TNIK, but not with the kinase domain (Shkoda et al, 2012). Association of TNIK with LMP1 is mediated by TRAF6, which is a direct interaction partner of TNIK. This furthermore underlines the importance of TRAF6 for the activation of JNK and canonical NF-κB (Shkoda et al, 2012).

1.6.2 The ERK and p38/MAPK Pathways

Mutational analysis with LMP1-transfected Rat-1 cells revealed that ERK activation could be abolished by complete mutation of the TRAF-binding motif in CTAR1 (P_{204}QQAT → A_{204}QAAA), but not by an A_{204}QAAT-mutant (Mainou et al, 2007). The same study showed that dominant-negative TRAF2 and TRAF3 also reduced ERK phosphorylation by LMP1, which would conclude that ERK1/2 is activated through CTAR1 in a TRAF2/3-dependent manner. It is, however, possible, that this CTAR1-induced ERK activation is not ras-dependent, since no activated ras precipitated with the ras-binding-domain of c-Raf from LMP1-expressing epithelial cells (Dawson et al, 2008). Also, immunoprecipitation studies showed that TRAFs 1 and 2 are not recruited to LMP1 with the mutations A_{204}QQAT or P_{204}QAAT (Devergne et al, 1998). Since the mutant A_{204}QAAT was still able to induce ERK (Mainou et al, 2007), it is unlikely that ERK activation is strictly depending on TRAF-binding to LMP1. A recent study additionally showed that expression of the CTAR2 domain alone is also capable of activating ERK in HEK293 cells

(Gewurz et al, 2011). This suggests that ERK1/2 activation can be regulated by LMP1 through different mechanisms, which might depend on the cell type used, but also on the integrity of the LMP1 protein.

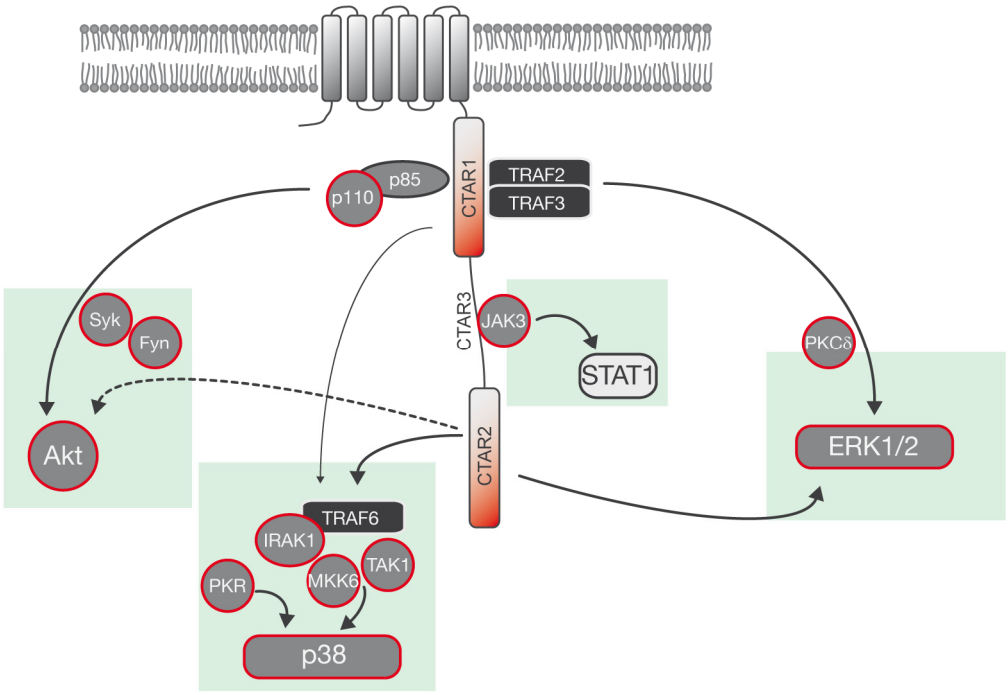

Figure 1-6. Schematic overview of LMP1-induced activation of PI3K/Akt, p38/MAPK, ERK1/2 and STAT pathways. The PI3K/Akt pathway is mainly induced by CTAR1, but CTAR2 can contribute. Syk and Fyn play important roles in the activation of Akt. The MAP-kinases p38 and ERK1/2 can be activated by both CTAR1 and CTAR2. Induction of the p38/MAPK pathway relies critically on TRAF6, and ERK1/2 activation is linked to the TRAF-binding site of CTAR1. STAT1 can be directly activated by binding of JAK3 to CTAR3.

Early studies showed that LMP1 activates the p38/MAPK pathway to induce IL-6 and IL-10, and that the activation of p38 kinase activity depends both on CTAR1 and to a slightly greater extend on CTAR2 (Eliopoulos et al, 1999b; Schultheiss et al, 2001). It was further demonstrated that TRAF6 plays an essential role in the activation of p38, since dominant negative TRAF6 lacking the RING domain blocks p38 activation by LMP1 (Schultheiss et al, 2001). Additionally, ectopic expression of TRAF6 could rescue defective p38 activation by LMP1 in TRAF6$^{-/-}$ MEFs (Schultheiss et al, 2001). Similarly, cells lacking IRAK-1 failed to induce phosphorylation of p38 upon expression of LMP1 (Song et al, 2006). Upstream kinases of p38 in LMP1 signaling include MKK6 (Schultheiss et al, 2001), TAK1 (Wan et al, 2004) and PKR (Lin et al, 2010). In EBV-immortalized cells, LMP1 can enhance its own transcription through the p38/MAPK

pathway (Johansson et al, 2010), thereby possibly inducing a self-sustaining autoregulatory loop.

Figure 1-6 depicts a schematic overview of the LMP1-induced p38/MAPK and ERK pathways, among others.

1.6.3 Regulation of PI3K/Akt by LMP1

LMP1 was first associated with PI3K/Akt activation in 2003. A study by Dawson et al. demonstrated that the p85α subunit of PI3K could be immunoprecipitated with LMP1, although it remained unclear if the interaction is direct or indirect (Dawson et al, 2003). The same study revealed that stable expression of LMP1 led to increased and constitutive activation of the PI3K/Akt pathway, which caused actin-remodeling, and was attributed to the CTAR1 domain. Additionally, the authors showed that inhibition of PI3K by LY294002 reduced LMP1's transforming potential in Rat-1 cells, suggesting a role for PI3K/Akt in LMP1-induced cellular transformation (Dawson et al, 2003). These findings were confirmed in further studies, and LMP1-induced PI3K/Akt signaling was suggested to be of importance for enhancing growth and for evasion of apoptosis (Jeon et al, 2007; Mainou et al, 2005; Mei et al, 2007; Shair et al, 2007; Yang et al, 2009a). The precise mechanisms of Akt activation by LMP1 have not been fully elucidated thus far. It is commonly appreciated that the CTAR1-domain is responsible for Akt activation (Dawson et al, 2003; Mainou et al, 2005), and that the P_{204}xQxT motif plays a role in this process (Lambert & Martinez, 2007). However, overexpression of the CTAR2-domain alone in EBV-positive C666.1 cells seemed to enhance Akt phosphorylation comparable to CTAR1 alone or full length LMP1 (Shair et al, 2008). Therefore it is possible that Akt activation by LMP1 can be achieved by different mechanisms depending on the cell type used, and that CTAR2 can play an enhancing role. Using inducible NGFR-LMP1 receptors in human B cells, a very recent study demonstrated that the activation of the PI3K/Akt pathway depends on spleen tyrosine kinase (Syk) and the Src kinase Fyn (Hatton et al, 2012). However, no Syk could be precipitated with LMP1 and it remains unclear how LMP1, which lacks the classical ITAM motif usually needed for Syk activation, can activate Syk (Hatton et al, 2012). Furthermore, Syk- and Fyn-dependent phosphorylation of c-Cbl could provide for a binding site of p85α in LMP1-dependent PI3K/Akt activation (Hatton et al, 2012). A brief depiction of the LMP1-induced PI3K/Akt pathway is included in figure 1-6.

1.6.4 JAK/STAT Signaling by LMP1

LMP1 has been discussed to induce the activity of STATs by different means (figure 1-6). In 1999 Gires et al. found that LMP1 induces JAK3-dependent STAT1 activation in B cells and HEK293 cells. They also demonstrated that JAK3 binds to the CTAR3 domain of LMP1, which

contains box1/2 motifs (Gires et al, 1999). Later, however, it was shown that deletion of the CTAR3 domain did not impair LMP1's ability to induce tyrosine-phosphorylation of STAT3 and STAT5, and that only very low amounts of JAK3 precipitate with LMP1 regardless of CTAR3 (Higuchi et al, 2002). Since then other mechanisms by which LMP1 activates STATs have been proposed. Cytokines like IL-6 are induced by LMP1-dependent p38/MAPK signaling (Eliopoulos et al, 1999b) to activate STAT in an autocrine manner, and neutralization of IL-6 on LMP1-expressing CNE2 cells greatly reduced STAT3-Tyr705 phosphorylation (Chen et al, 2003). Interferon γ (IFN-γ) has also been associated with STAT activation by LMP1. Vaysberg et al. showed that in PTLD-derived B cells LMP1 activates STAT1 without directly interacting with JAK3, but rather by inducing the secretion of IFN-γ through NF-κB and p38/MAPK dependent gene transcription (Vaysberg et al, 2009). This means that activation of the JAK/STAT pathway by LMP1 can be achieved by indirect signaling mechanisms including autocrine activation loops, but the involved LMP1 domains and pathways are insufficiently characterized. It was further shown that LMP1-dependent phosphorylation of STAT3-Ser727 is mediated by ERK (Liu et al, 2008). It is possible that this is a downstream effect of CTAR1-mediated activation of PKCδ, because inhibition of PKCδ abolishes phosphorylation of both ERK and STAT3 (Kung et al, 2011).

Interestingly, STAT3 was also shown to specifically bind the LMP1 L1-TR-promoter and stimulate LMP1 expression (Chen et al, 2003; Chen et al, 2001). This proposes a mechanism, which secures LMP1 expression by exogenous cytokine stimuli, but also by self-sustaining autocrine signaling loops.

On the bottom line it is evident that although extensive research has unraveled some of the mechanisms involved in the LMP1-dependent activation of the signaling pathways described in the previous subchapters, many aspects of the LMP1 signaling network still remain controversial and insufficiently understood.

1.6.5 Aims of this Thesis

Despite great scientific efforts over the past 15 – 20 years investigating the exact mechanisms by which the oncoprotein LMP1 induces such a vast variety of signaling pathways, many aspects still remain unclear or controversial. As discussed in the previous chapters some of the acquired data concludes for conflicting results. This can be due to several factors. On the one hand, certain results seem to be linked to the cell type used and LMP1 seems to be able to induce signaling by different means in different cell types. Furthermore, a lot of data has been acquired using overexpression of TRAFs or TRADD or of dominant negative mutants of these molecules, and these data do not necessarily reflect the complete spectrum of possibilities LMP1 possesses to make use of these proteins. At the same time only very few studies have looked at the combined effect of TRAFs and TRADD with the different CTAR domains, especially not in one cellular system.

The present study set out to establish an experimental system to systematically investigate the mechanisms of LMP1 signaling. The goal was to investigate the effects of the deficiency of TRAFs and TRADD and mutation of single CTAR domains within a similar cellular context. Using the genetically clean background of knockout mouse embryonic fibroblasts (MEFs), this system would grant the possibility to decipher carefully what roles exactly the different TRAF and TRADD molecules play in LMP1-induced signaling pathways induced by single domains. All data would be acquired in MEF cell lines, resulting in a thorough and comprehensive picture of LMP1 signaling mechanisms within one cellular system, answering the question which signaling pathways are initiated at which LMP1 signaling domain through which cellular signaling molecule.

Furthermore, the aim was to use an inducible, chimeric NGFR-LMP1 receptor rather than a native, constitutively active molecule. This provides an important advantage. Constitutive long term signaling naturally leads to the induction of secondary signaling pathways and to the intrinsic self-regulation of active signaling pathways. This undoubtedly blurs the resulting picture to a certain extent, since primary and secondary signaling effects would not be discernible any more. Using an inducible receptor the precise kinetics of signaling can be studied. This provides the opportunity to discriminate primary signaling events form secondary effects.

The new insights into LMP1 signaling expected to be acquired from this systematical study would reveal new aspects of LMP1 signaling that can provide a basis for further, in depth studies concerning interesting aspects of LMP1 signaling.

2 MATERIALS

2.1 Chemicals, Reagents, Equipment

2.1.1 Inhibitors

NF-κB Activation Inhibitor II, JSH-23	Calbiochem	481408
JNK inhibitor SP600125	Enzo	BML-EI305
p38 inhibitor SB203580	Adipogen	AG-CR1-0030
cycloheximide	Calbiochem	239763
MG-132	Calbiochem	474791
complete Mini protease inhibitor mix	Roche	11 836 153 001

2.1.2 Ligands

TNFα (human)	Roche	11 371 843 001
rHu IGF-1	Applichem	A8530

2.1.3 Chemicals, Reagents, Media

Standard laboratory chemicals were purchased from Sigma, Applichem, Merck or Roth

dNTPs	Roche
GneRuler DNA ladder mix	Fermentas Life Sciences
VENT® DNA-Polymerase, Thermopol buffer	New England Biolabs
restriction enzymes and reaction buffers	New England Biolabs
T4 DNA ligase and T4 ligation buffer	New England BIolabs
LightCycler® 480 SYBR Green I Master	Roche
ampicillin / kanamycin	Sigma
DMEM / RPMI 1640 cell culture media	Gibco®, Invitrogen
fetal calf serum (FCS)	Gibco®, Invitrogen
penicillin/streptomycin for cell culture	Gibco®, Invitrogen
Trypsin-EDTA	Gibco®, Invitrogen
DMSO	Roth
PolyFect® transfection reagent	Qiagen
NP-40 (igepal)	Sigma
Bradford reagent	BioRad
PageRuler Plus Prestained Protein Ladder	Fermentas Life Sciences
Optiprep™	Sigma
Cholera Toxin Subunit B Conjugate	Molecular Probes MP34775
Tween-20	Amershan Pharmacia Biotech

2.1.4 Equipment

test tubes 0.5 / 1.5 / 2 ml	Eppendorf
test tubes 15 / 50 ml (Falcon)	Beckton Dickinson
cryotubes 1.5 ml	Thermo Fisher Scientific
cell culture dishes, multiwell plates	Thermo Fisher Scientific
cell culture flasks	Greiner
micropipettes, pipette tips	Gilson
sterile pipette tips with filter	Kisker
PCR test tubes	Life Technologies
Robocycler Gradient 96	Stratagene
Perfect Blue agarose gel system	Peqlab
96-well plates for qRT-PCR	4titude
LightCycler® 480	Roche

GenePulser II, electroporation cuvettes	BioRad
FACS Calibur	Beckton Dickinson
fluorescence microspcope	Zeiss
Leica TCS SP5 II confocal microscope	Leica
PerfectBlue Vertical Double Gel System	Peqlab
PerfectBlue Semi-dry electroblotter	Peqlab
Nitrocellulose membrane Protran BA79 (0.1 µm)	GE Healthcare/Whatman
Optimax developing machine	Typon Medical
X-ray films CEA Blue Sensitive	AGFA Healthcare
Centrifuges	Eppendorf, Heraeus

2.2 Antibodies

2.2.1 Primary Immunoblot Antibodies

Target	Company	
Akt	Cell Signaling	#9272
P-Akt (Ser473) (D9E) XP	Cell Signaling	#9270
ERK1/2 (p44/p42 MAPK)	Cell Signaling	#9102
P-ERK1/2 (Thr202/Tyr204)	Cell Signaling	#9101
IκBα (C-21)	Santa Cruz	sc-371
P- IκBα (Ser32)	Cell Signaling	#9246
JNK1 (C-17)	Santa Cruz	sc-474
P-JNK1/2/3 (Thr183/Tyr185)	abcam	ab59196
LMP1 (3G6-1)	HMGU E. Kremmer	
p100/p52	Cell Signaling	#4882
p38α/β (H-147)	Santa Cruz	sc-7149
P-p38 (Thr180/Tyr182) (D3F9) XP	Cell Signaling	#4511
STAT3 (H-190)	Santa Cruz	sc-7179
P-STAT3 (Tyr705) (D3A7) XP	Cell Signaling	#9145
P-STAT3 (Tyr705) (9E12)	Upstate/Millipore	#05-485
TRADD (H-278)	Santa Cruz	sc-7868
TRAF2 (C-20)	Santa Cruz	sc-876
TRAF3 (C-20)	Santa Cruz	sc-949

TRAF6 (H274)	Santa Cruz	sc-7221
αTubulin (B-5-1-2)	Santa Cruz	sc-23948

2.2.2 Secondary Immunoblot Antibodies

anti-mouse HRP-linked	Cell Signaling	#7076
anti-rabbit HRP-linked	Cell Signaling	#9270
anti-rat HRP-linked	Jackson/dianova	#112-035-167

2.2.3 Crosslinking Antibodies

NGFR	HMGU E. Kremmer	HB8737
IgG/IgM (H+L) goat anti-mouse	Jackson/dianova	115-005-068

2.2.4 Neutralizing Antibodies

mouse LIF	R&D Systems	AB-449-NA
mouse IL-6	R&D Systems	MAB406
mouse M-CSF	R&D Systems	MAB4161
mouse CXCL1	R&D Systems	MAB4531
mouse epiregulin (EREG)	R&D Systems	MAB1068
mouse GM-CSF Rα	R&D Systems	MAB6130

2.2.5 Antibodies for Immunofluorescence and FACS

Alexa Fluor 647 anti-CD271 (NGFR)	BD Pharmingen	560877
Cy3 anti-mouse IgG (H+L)	Jackson/dianova	115-165-166
anti-LMP1 (CS.1-4)	Dako Cytomation	M 0897

2.3 Primers

2.3.1 Primers for PCR

AK322 5‘-GGGTAGGCGGCCGCACCATGGGGGCAGGTGCCACCGGCCGCGCC ATGGAC

AK323	5'- GGGGAGTGGCGGCCGCACCGGTTTAGTCATAGTAGCTTAGCTGAACTGG
AK324	5'-GGGGAGTGGCGGCCGCATGCATTAGTCATAGCCGCTTAGCTGAACTGG
AK341	5'- GGGGGGGTCCGGACTTGTACAGCTCGTCCATGCCGAGAG
AK352	5'-CACGGGGACGTGGTTTCCCTTTGAAAAACACGATAATACCATGGTGAGCAAGGGCGAGGAGCTG
AK377	5'- GGGCGGGGCGGCCGCACCATGGACTACAAAGACGATGACGAC
AK378	5'-GGGCGGGGCGGCCGCCTCGAGTTACTATACCCCTGCATCAGTACTTCG
P1 fwd	5'-GGGTCATGCCACTTGTTCGA
P2 rev	5'-ACCCACACGAGGAAGGTCTGA

2.3.2 Primers for qRT-PCR

human HPRT	fwd	5'-TGACCTTGATTTATTTTGCATACC
	rev	5'- CGAGCAAGACGTTCAGTCCT
murine HPRT	fwd	5'-TCCTCCTCAGACCGCTTTT
	rev	5'-CCTGGTTCATCATCGCTAATC
LMP1	fwd	5'-TCCTCCTCTTGGCGCTACTG
	rev	5'- TCATCACTGTGTCGTTGTCC

Commercially Available

QuantiTect® Primer Assay (Qiagen)

murine	human
Mm_Tgfb1_1_SG	Hs_FGF7_2_SG
Mm_Ngf_1_SG	Hs_CXCL1_1_SG
Mm_Gdf5_1_SG	Hs_Il6_1_SG
Mm_Fgf9_1_SG	Hs_CSF1_1_SG
Mm_Fgf7_2_SG	Hs_EREG_1_SG
Mm_Fgf4_2_SG	Hs_LIF_1_SG
Mm_Vegfc_1_SG	Hs_CSF3_1_SG
Mm_Lif_1_SG	Hs_CSF2_1_SG
Mm_Il6_1_SG	
Mm_Il3_1_SG	
Mm_Il12α_1_SG	

Mm_Il11_1_SG

Mm_Il1α_1_SG

Mm_Igf1_2_SG

Mm_Ereg_1_SG

Mm_Cxcl1_1_SG

Mm_Csf3_1_SG

Mm_Csf2_1_SG

Mm_Csf1_2_SG

2.4 Plasmids

pCMV-HA-LMP1wt – pHEBO expression vector for HA-tagged (N-term.) LMP1wt (aa 6-386) from the EBV B95.8 strain under the control of a CMV promoter (Schneider et al, 2008)

pCMV-HA-LMP1(A_{204}xAxA) – pHEBO expression vector for HA-tagged (N-term.) LMP1 with a P_{204}xQxT → A_{204}xAxA mutation in CTAR1 under the control of a CMV promoter (Schneider et al, 2008)

pCMV-HA-LMP1(Y_{384}G) – pHEBO expression vector for HA-tagged (N-term.) LMP1 with a Y_{384}G mutation in CTAR2 under the control of a CMV promoter (Schneider et al, 2008)

pCMV-HA-LMP1(Δ371) – pHEBO expression vector for HA-tagged (N-term.) LMP1 with a deletion of the last 16 C-terminal amino acids of CTAR2 under the control of a CMV promoter (Schneider et al, 2008)

pCMV-HA-LMP1(A_{204}xAxA/Y_{384}G) – pHEBO expression vector for HA-tagged (N-term.) LMP1 with a P_{204}xQxT → A_{204}xAxA mutation in CTAR1 and a Y_{384}G mutation in CTAR2 under the control of a CMV promoter (unpublished, provided by A. Kieser)

pCMV-HA-LMP1(A_{204}xAxA/Δ371) – pHEBO expression vector for HA-tagged (N-term.) LMP1 with a P_{204}xQxT → A_{204}xAxA mutation in CTAR1 and a deletion of the last 16 C-terminal amino acids of CTAR2 under the control of a CMV promoter (Schneider et al, 2008)

p1768 (3xκB-Luc) – luciferase reporter plasmid with three NF-κB binding sites (Mitchell & Sugden, 1995)

pPGK-Renilla – Renilla-Luciferase reporter plasmid under the control of a PGK promoter

pEGFP-C1 – expression vector for enhanced GFP under CMV promoter control (GenBank Accession # U55763)

pGagPol – expression plasmid for retroviral gag/pol proteins for packaging of retroviruses (Morita et al, 2000)

pEnv - expression plasmid for ecotrophic retroviral env glycoproteins for packaging of retroviruses (Morita et al, 2000)

SF91-IRES-GFP-WPRE ("3054") – retroviral expression vector with LTR-sequences flanking unique NotI cloning site followed by IRES-GFP and gWPRE (Schwieger et al, 2002)

3054-IRESeCFP – retroviral SF91-IRES-GFP-WPRE vector, in which GFP was replaced by CFP by insertion of a PCR fragment generated from pRK5_eCFP-hTRAF2(M246) with primers AK352 and AK341 via BmgBI and BspEI (cloned in the course of this work)

pRK5_eCFP-hTRAF2(M246) – pRK5 expression vector encoding ECFP-hTRAF2 fusion protein starting at M246 of original TRAF2 sequence (unpublished, provided by A. Kieser)

pRK5-Flag-TRAF6 – pRK5 expression vector for wildtype human TRAF6 with an N-terminal Flag-tag (Shkoda et al, 2012)

1755.1 NGFR-LMP1wt – expression vector for NGFR-LMP1wt driven by SV40 promoter in pHEBO vector, additionally encoding hygromycin B phosphotransferase (Gires et al, 1999)

pSV-NGFR-LMP1(PQT->AAA) – expression vector for SV40-promoter driven NGFR-LMP1 with a P_{204}xQxT → A_{204}xAxA mutation in CTAR1, based on pHEBO vector (Kieser et al, 1999)

pSV-NGFR-LMP1(Y_{384}G) – expression vector for SV40-promoter driven NGFR-LMP1 with a Y_{384}G mutation in CTAR2, based on pHEBO vector (Kieser et al, 1999)

pSV-NGFR-LMP1(PQT/Y_{384}G) – expression vector for SV40-promoter driven NGFR-LMP1 with a P_{204}xQxT → A_{204}xAxA mutation in CTAR1 and a Y_{384}G mutation in CTAR2, based on pHEBO vector (Kieser et al, 1999)

3054-NGFR-LMP1wt – retroviral expression vector based on SF91-IRES-GFP-WPRE; NGFR-LMP1wt-sequence was cloned into the vector via NotI from PCR-fragment generated from template vector 1755.1NGFR-LMP1wt with primers AK322 and AK323 (cloned in the course of this work)

3054-NGFR-LMP1(A_{204}xAxA) - retroviral expression vector based on SF91-IRES-GFP-WPRE; NGFR-LMP1(A_{204}xAxA)-sequence was cloned into the vector via NotI from PCR-fragment generated from template vector pSV-NGFR-LMP1(PQT->AAA) with primers AK322 and AK323 (cloned in the course of this work)

3054-NGFR-LMP1(Y_{384}G) - retroviral expression vector based on SF91-IRES-GFP-WPRE; NGFR-LMP1(Y_{384}G)-sequence was cloned into the vector via NotI from PCR-fragment generated from template vector pSV-NGFR-LMP1(Y_{384}G) with primers AK322 and AK324 (cloned in the course of this work)

3054-NGFR- LMP1(A_{204}xAxA/Y_{384}G) - retroviral expression vector based on SF91-IRES-GFP-WPRE; NGFR-LMP1(A_{204}xAxA/Y_{384}G)-sequence was cloned into the vector via NotI from PCR-fragment generated from template vector pSV-NGFR-LMP1(PQT/Y_{384}G) with primers AK322 and AK324 (cloned in the course of this work)

3054-Flag-TRAF6wt-eCFP – retroviral expression vector expressing Flag-tagged human TRAF6wt based on SF91-IRES-GFP-WPRE; TRAF6wt sequence was cloned into the vector 3054-IRESeCFP via NotI from PCR-fragment generated from template vector pRK5-Flag-TRAF6 with primers AK377 and AK378. (cloned in the course of this work)

2.5 Eukaryotic Cell Lines

wildtype MEF	mouse embryonic fibroblasts, littermate strain to TRADD$^{-/-}$ MEFs (Ermolaeva et al, 2008)
TRADD$^{-/-}$ MEF	mouse embryonic fibroblasts with a homozygous knockout for TRADD (Ermolaeva et al, 2008)
TRAF6$^{-/-}$ MEF	mouse embryonic fibroblast with a homozygous deletion of TRAF6 (Lomaga et al, 1999)
TRAF2/5$^{-/-}$ MEF	mouse embryonic fibroblast with homozygous deletions of TRAF2 and TRAF5 (Tada et al, 2001)
CNE-L	human nasopharyngeal carcinoma cell line that is EBV-negative (Thomas et al, 2003)
P493-6	human LCL cell line based on EREB 2-5 cells (Kempkes et al, 1995), which stably carries a tetracyclin-regulated c-myc expression vector (Pajic et al, 2000; Schuhmacher et al, 1999)
Phoenix (ΦNXgp)	HEK293 cells especially suitable as retrovirus packaging cells, expressing gag/pol of MLV (Pear et al, 1993)

2.6 Prokaryotic Cell Lines

DH5α	(Invitrogen, Life Technologies Corp.) K-12 F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) gal- phoA supE44 λ- thi-1 gyrA96 relA1
GM2163	(Thermo Fisher Scientific) F^{-} dam-13:Tn9(Cam^{R}) dcm-6 ara-14 hisG4 leuB6 thi-1 lacY1 galK2 galT22 glnV44 hsdR2 xlyA5 mtl-1 rpsL 136(Str ^{R}) rtbD1 tonA31 tsx78 mcrA mcrB1

2.7 Commercially Available Kits

Jetstar 2.0 Plasmid Maxi Prep Kit	Genomed
NucleoSpin® Extract II kit	Machery-Nangel
Dual-Luciferase® Reporter Assay System	Promega
QuantiTect® Reverse Transcription Kit	Qiagen
QIAamp DNA Mini Kit	Qiagen
RNeasy mini kit	Qiagen
RT^2 Profiler PCR Array and First Strand kit	Qiagen

2.8 Software

Mac OSX

Windows 8

Microsoft Office 2012

Mac Vector 11

Flow Jo vX

GraphPad Prism 5

Adobe CS3 & CS4

EndNote X6

3 METHODS

3.1 Molecular Biology

3.1.1 Polymerase Chain Reaction (PCR)

The PCR was prepared in 50 µl reaction volume with 10-100 ng template DNA, 0.2 mM dNTP Mix, 1 x Thermopol buffer, 100 pmol of each rev and fwd primer, and 1 U Vent DNA-Polymerase. The reaction was performed according to the following standard protocol:

1 x cycle	initial denaturation	10 min	95 °C
33 x cycles	denaturation	1 min	95 °C
	annealing	1 min	58 °C
	elongation	2 min	72 °C
1 x cycle	final elongation	10 min	72 °C

dNTP Mix 20 mM dATP, 20 mM dCTP, 20mM dGTP, 20 mM dTTP in H_2O

3.1.2 Agarose Gel-Electrophoresis

To separate DNA-fragments by their respective size the DNA-samples were mixed with DNA loading dye and loaded onto a 1 – 2 % (w/v) agarose gel to be run in 1 x TBE buffer at 20 – 120 V for 3 – 16 h. Due to ethidiumbromide staining the DNA bands could be visualized under UV light.

If PCR-products or DNA-fragments of a desired size were needed for further experiments, they were cut out of the gel matrix and cleaned with the NucleoSpin Extract II kit according to the manufacturer's instructions.

DNA loading dye	10 % (v/v) glycerol, 0.004 % bromphenol blue in TBE buffer
TBE buffer pH 8	89 mM Tris, 89 mM boracic acid, 2mM EDTA (ethylenediaminetetraacetic acid)

3.1.3 Restriction Digestion

Restriction digestion of PCR-products or plasmid DNA was performed in 100 µl reaction volume with 1 U of the designated enzyme per 1 µg DNA, and the fitting reaction buffer according to the manufacturer. The reaction was conducted at the appropriate temperature for 15 min to 1 h. Afterwards the fragments could be seperated by agarose gel electrophoresis.

3.1.4 Ligation of DNA-Fragments

To ligate digested DNA-fragments into vector plasmids, 100 ng plasmid DNA and a 5 – 10-fold molar excess of insertable DNA-fragments were mixed with 1 x T4 ligation buffer and 1 U T4 DNA ligase in 15 µl reaction volume and incubated overnight in a 16 °C waterbath.

3.1.5 Plasmid Preparation

Mini-Preparation

Small amounts of DNA for analysis purposes were prepared with a down-scaled protocol using the solutions from Jet Star Maxi Kit. The bacteria were inoculated into 5 ml of LB medium supplemented with the appropriate antibiotic and incubated overnight at 37 °C in a bacteria shaker. 1 ml of the bacteria culture was transferred into a 1.5 ml test tube and centrifuged at 3000 rpm for 15 min at RT. The pellet was resuspended in 200 µl Jet Star E1 buffer supplemented with RNAse, before adding 200 µl E2 lysis buffer and mixing gently for 5 min. Afterwards the lysis was stopped by adding 200 µl of E3 neutralization buffer and mixing thoroughly before centrifugation at 16000 x g for 30 min at RT. The supernatant was transferred

to a fresh 1.5 ml test tube and the DNA was precipitated by adding 0.7 x vol Isopropanol and centrifuging for 30 min at 16000 x g. The DNA pellet was air dried and eluted in 20 – 30 µl of H_2O.

Maxi-Preparation

The protocol and kit for large scale DNA-preparation from Jet Star was used to prepare large amounts of plasmid DNA from bacteria. 400 ml LB medium supplemented with the appropriate antibiotic were inoculated with bacteria from culture plates or freezer stock and incubated overnight at 37 °C in a bacteria shaker. After centrifugation at 3000 x g for 15 min at RT the bacteria pellet was resuspended in 10 ml E1 buffer supplemented with RNAse and transferred into 50 ml ultracentrifugation tubes. 10 ml E2 lysis buffer was added to the suspension and mixed very gently for 5 min, before stopping the lysis with 10 ml E3 neutralization buffer and centrifugation at 15000 x g for 15 min at RT. The supernatant was then transferred into an anion exchange column (provided by the kit) equilibrated with 30 ml E4 equlibration buffer. After washing with 60 ml E5 washing buffer, the DNA was eluted from the column by adding 15 ml E6 eluation buffer and collecting the flow through in a fresh 50 ml Falcon tube. The DNA was precipitated by adding 10.5 ml isopropanol and centrifugation at 5000 rpm for 50 – 90 min at 4 °C. The DNA pellet was washed once with 80% Ethanol and air dried before dissolving in 400 µl H_2O.

3.1.6 Extraction of Genomic DNA from Cells

1 x 10^6 cells were harvested and washed once with 1 x PBS. Preparation of genomic DNA was conducted with the help of the “QIAamp DNA mini” kit following the instructions provided by the manufacturer (11/2007).

1 x PBS	137 mM NaCl, 2,7 mM KCl, 10 mM Na_3PO_4, 2 mM K_3PO_4

3.1.7 Quantitative RT-PCR (qRT-PCR)

Preparation of RNA from Cells

Total RNA extraction was performed at RT using the RNeasy kit from Qiagen according to the manual (January 2011). In short, cells were washed once with 1 x PBS and lysed by vortexing the samples shortly with 350 µl RLT buffer. After thorough resuspension with 350 µl 70% EtOH, the lysate was loaded onto a spin column supplied with the kit and centrifuged at 8000 x g for 15 sec. The flowthrough was discarded and 700 µl RW1 buffer were added to the column. After centrifugation the column was washed twice with 500 ml RPE buffer, then the RNA was eluted

by adding 25 – 50 µl RNAse free H_2O to the dried column membrane and centrifuging for 1 min at 8000 x g. The flow through was collected in a fresh 1.5 ml reaction tube and the RNA concentration was determined with the help of a NanoDrop. The RNA samples were stored at -80°C for long time storage.

cDNA Synthesis

RNA was transcribed to cDNA by using the Qiagen QuantiTect Reverse Transcription kit according the manual (March 2009). Exactly 1 µg of RNA was used for each reaction and incubated for 2 min at 42 °C in a total volume of 12 µl with 2 µl genomic DNA wipeout buffer to get rid of any genomic DNA in the sample. Afterwards 4 µl RT reaction buffer, 1 µl Primer Mix and 1 µl RT were added to the reaction, which was incubated for 15 min at 42 °C. The reaction was stopped by inactivating the RT for 3 min at 95 °C. cDNA samples were kept at -20 °C for long time storage.

RT^2 Profiler PCR Array

The Qiagen RT^2 Profiler PCR Array is a q RT-PCR based array to screen for a large amount of target genes in one assay. RNA for this array was prepared as described in *3.1.6.1,* and cDNA was generated using the Qiagen RT^2 First Strand kit according to the manual. The acquired cDNA was used in the RT^2 Profiler PCR Array following the instructions provided by the manufacturer. The PCR reaction and analysis were performed using the Roche LightCycler 480 with a program setup as follows:

1) activation	1 cycle	10 min	95 °C	ramp rate 4.4
2) amplification	45 cycles	15 sec	95 °C	ramp rate 4.4
		1 min	60 °C	ramp rate 2.2
		single acquisition after each cycle		
3) melting curve	1 cycle	1 min	95 °C	ramp rate 4.4
		2 min	65 °C	ramp rate 2.2
		hold	97 °C	ramp rate 0.11
		continuous acquisition per 5 °C		
4) cooling	1 cycle	10 sec	40 °C	ramp rate 2.2

Relative induction levels of mRNAs were calculated using the light cycler software analysis function.

qRT-PCR Reaction

qRT-PCR reactions were used to do relative quantifications of certain mRNA levels in cells subjected to different stimuli. Before use the generated cDNA was diluted with 80 µl H_2O to a total volume of 100 µl. The primers for the targets (Qiagen QuantiTect Primer Assay), which were stored at 100 x concentration, were further diluted to 10 x prior to use in the assay. Forward and reverse primers for hprt or LMP1 with a stock concentration of 100 nmol were mixed at a 1:1 ratio and diluted 1:10 with H_2O prior to use. The qRT-PCR reaction mix was pipetted into one well of a 96-well PCR plate, sealed with a translucent cover film, and the PCR reaction was carried out in a Roche LightCycler 480 machine. The C_T values were calculated with the help of the LightCycler software, and the relative induction levels were calculated using the $2^{-\Delta\Delta C_T}$ method as described by Livak and Schmittgen (Livak & Schmittgen, 2001).

qRT-PCR reaction mix 1 µl cDNA, 5 µl SYBR Green I Master, 1 µl primer mix (0.5 µl primer mix hprt), 3 µl H_2O (3.5 µl H_2O hprt)

LightCycler program setup

1) pre-incubation	1 cycle	15 min	95 °C	ramp rate 4.4
2) amplification	45 cycles	15 sec	95 °C	ramp rate 4.4
		30 sec	55 °C	ramp rate 2.2
		30 sec	72 °C	ramp rate 4.4
		single acquisition after each cycle		
3) melting curve	1 cycle	1 sec	97 °C	ramp rate 4.4
		10 sec	67 °C	ramp rate 2.2
		hold	97 °C	ramp rate 0.11
		continuous acquisition per 5°C		
4) cooling	1 cycle	15 sec	37 °C	ramp rate 2.2

3.2 Microbiology

3.2.1 Culturing and Storage of Bacteria

For large scale progeny bacteria were grown in suspension culture. The desired volume of LB medium was inoculated with bacteria either from a frozen stock culture or from an agar plate and incubated overnight in a 37 °C bacteria shaker. If selection was desired, the medium was supplemented with the appropriate antibiotic.

For selection of single clones bacteria were cultivated on LB agar plates supplemented with the appropriate antibiotic overnight in a 37 °C incubator.

To store bacteria for long time periods, cryo vials filled with a dense suspension culture with 15% glycerol were suspended in liquid nitrogen for shock freezing and stored at -80 °C.

LB medium	1 % (w/v) Trypton, 0.5 % (w/v) yeast-extract, 0.5 % (w/v) sodium chloride
ampicillin	fc 100 µg/ml
kanamycin	fc 30 µg/ml

3.2.2 Transformation of Bacteria

The protocol for generation and transformation of chemically competent bacteria was adapted from the Hanahan method (Hanahan, 1983).

Generation of Chemically Competent Bacteria

E.coli of the strains DH5α or GM2163 were inoculated into 5 ml SOB medium from frozen stocks and incubated overnight in a 37 °C bacteria shaker. 1 ml of the overnight culture was transferred into 50 ml prewarmed SOB medium and incubated at 37 °C until the OD_{600} was between 0.3 and 0.55. 25 ml of the bacteria culture was transferred into a 50 ml Falcon and incubated on ice for 15 min. After centrifugation for 5 min at 3000 rpm and 4 °C the pellet was resuspended in 8.3 ml ice cold TFB buffer and incubated for another 15 min on ice. After another centrifugation step (5 min, 3000 rpm, 4 °C) the pellet was resuspended in 2 ml ice cold TFB buffer and left on ice for 15 min. After addition of 70 µl DMSO the bacteria were divided into 200 µl aliquots and used right away for transformation or shock frozen in liquid nitrogen to keep at -80 °C for long time storage, after addition of 85 µl of 50% glycerin.

SOB medium	LB medium with 10 mM $MgSO_4$ and 10 mM $MgCl_2$
TFB buffer	10 mM MES (pH 6.3), 45 mM $MnCl_2$ x 4 H_2O, 10 mM $CaCl_2$ x 2 H_2O, 100 mM KCl, 3 mM hexamine cobalt chloride in 1 l H_2O

Transformation of Chemically Competent Bacteria by Heat Shock

15 µl DNA from a ligation step or 200 – 500 ng Plasmid DNA were added into 200 µl competent bacteria in a 1.5 ml test tube and incubated for 30 min on ice. The heat shock was carried out for exactly 2 min at 42 °C before the samples were returned to ice for another 2 min. Afterwards

1 ml LB medium was added and the samples were incubated in a 37 °C shaker for at least 30 min. After centrifugation at 3000 rpm for 5 min at RT the bacteria pellet was resuspended in 50 - 100 µl LB medium and plated on LB agar plates, which were cast with the appropriate antibiotic for selection, to incubate overnight at 37 °C.

3.3 Cell Biology

3.3.1 Culturing Cells

Cells were cultured in complete medium on cell culture dishes (adherent cells) or in flasks (suspension cells) and kept at 37 °C with 5% CO_2. Cells were passaged at 70% confluence for adherent cells, or 1 x 10^6 cells/ml for suspension cells. MEFs were cultured in complete DMEM medium and Phoenix, CNE or P493-6 cells were kept in complete RPMI medium. Some cell line also required the addition of special supplements, as listed below.

complete medium	10% FCS, 1 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin in DMEM or RPMI
special supplements	*CNE:NGFR-LMP1wt*: 100 µg/ml hygromycin B
	P493-6 ER/EB: 1 µM estrogen, 0.1 µg/ml doxycyclin
	Phoenix: 10 mM HEPES

3.3.2 Freezing Cells for Long Term Storage

Cells were prepared for storage by centrifugation at 900 rpm for 5 min. The cell pellet was resuspended in 1 ml FCS with 15% (v/v) DMSO, before 1 ml complete medium was added. The cell suspension was transferred to a cryo vial and left at -80 °C in a freezing box with isopropanol for a gentle cool down over 1 – 2 days. Afterwards the vial was resuspended in liquid nitrogen for long time storage.

3.3.3 Stimulation of Cells

NGFR-Crosslinking

For NGFR-crosslinking the desired amount of cells was seeded in one cell culture dish per anticipated time point and starved in minimal medium without FCS for 16-24 h after adherence was complete. To prepare for crosslinking the cells were stained for 1 h at 37 °C in minimal medium containing 1 µg/ml anti-NGFR antibody. Thereafter the cells were quickly washed once with fresh minimal medium and incubated with minimal medium containing 10 µg/ml anti-fc IgG/IgM crosslinking antibody for the designated time periods ranging from 20 min to 10 h. At

the end of each crosslinking period the cells were quickly washed once with 1 ml ice cold 1 x PBS and lysed to be used for experiments.

culture dish	number of cells	stimulation medium
3 cm	2 x 10^5	1 ml
6 cm	5 x 10^5	1.5 ml
10 cm	1.5 x 10^6	5 ml
15 cm	3.5 x 10^6	15 ml

All experiments were performed such that a specific NGFR-LMP1 mutant or knockout of a signaling molecule was analyzed in direct comparison with the wt MEF:NGFR-LMP1wt cells. This procedure aimed at achieving maximum comparability between wildtype and mutant or knockout status, because it was possible that different experiments slightly vary with regard to the stimulation or immunoblotting procedures.

Usage of Soluble Ligands and Inhibitors

For stimulation with TNFα or IGF-1 the cells were seeded in 6 cm dishes as described above and starved overnight in minimal medium. To stimulate the cells the medium was aspirated and 1.5 ml minimal medium supplemented with 20 ng/ml TNFα or IGF-1 was quickly added to the cells to incubate for the designated time at 37 °C. Afterwards the cells were immediately washed once with 1 ml ice cold 1 x PBS on ice to stop the stimulation.

Different chemical compounds were used to inhibit certain cellular processes during NGFR-crosslinking. For this, all crosslinking media were supplemented with the desired amount of inhibitor. This way the cells were pre-incubated with the inhibitors for one hour prior to the crosslinking period during α-NGFR binding, while fresh inhibitor was still present during the crosslinking periods.

Treatment of Cells with Cell Culture Supernatant

Target cells were seeded in one 6 cm cell culture dish per time point at a density of approximately 5 x 10^5 cells per dish and starved for 16 – 24 h in minimal medium. For treatment the supernatant (SN) was harvested from the producer cells and either kept at 37 °C until usage or used right away to treat the target cells. Every 6 cm dish received 1.5 ml of supernatant and was incubated for 20 or 30 min at 37 °C.

For neutralization of soluble factors the supernatant was incubated with 10 µg/ml of neutralizing antibodies against the designated target factor for 30 min rolling at RT followed by another 30 min at 37 °C before the supernatant was added to the target cells.

3.3.4 Transfection of Eukaryotic Cells

Electroporation

Electroporation is used to transfect cells with DNA by applying an electrical field to the cell and thereby increasing the permeability of the plasma membrane. 1 x 10^7 MEFs were prepared by centrifugation at 900 rpm for 5 min and resuspension of the pellet in 250 µl minimal DMEM. A total amount of 20 µg plasmid DNA was added to the cells to be incubated at RT for 15 – 20 min. The cell suspension was transferred into an electroporation cuvette with 4 mm electrode gap and electroporated with the BioRad GenePulser II machine at 240 V and 0.975 mFa. Immediately afterwards 500 µl pre-warmed FCS was added to the cells and they were transferred to a 6-well plate with 2 ml complete DMEM to grow at 37 °C and 5% CO_2 for 24 h.

Liposome Transfection

Liposome transfection can be used to introduce DNA into a cell. The DNA is packed in liposomes that fuse with the cell membrane to release its contents into the cytosol. CNE-L cells were seeded at 60% confluency and left to adhere. The cells were washed once with minimal RPMI medium before the transfection. The DNA and 2 µl Polyfect per 1 µg DNA were dissolved separately in 100 µl minimal RPMI. Both solutions were mixed and incubated at RT for 15 min for liposome formation. The transfection mix was added into minimal RPMI and carefully administered to the prepared cells to incubate for 5 h at 37 °C and 5% CO_2. Afterwards the medium was changed to fresh minimal RPMI for overnight incubation.

3.3.5 Hygromycin Selection and Generation of Single Cell Clones

To select for hygromycin resistant clones CNE-L cells were seeded at low density (10 – 20% confluency) in large cell culture dishes one day after transfection. After adherence was complete, the medium was changed to complete RPMI medium containing 100 µg/ml hygromycin B, and the cells were grown over several weeks at 37°C and 5% CO_2, until hygromycin resistant single cell clonal colonies were observable. To separate individual clonal populations from the plate, sterile filter paper snippets soaked in Trypsin were carefully placed on top of one colony and left for incubation for 3 to 5 minutes. Afterwards the detached cells were carefully swiped off the plate using the filter paper and sterile tweezers. The filter paper along with the cells was transferred to a 12-well plate containing 1 ml fresh complete RPMI medium containing 100 µg/ml hygromycin B. The single clone cell lines were cultivated as described in chapter 3.3.1 in the presence of hygromycin B.

3.3.6 Preparation of Infectious Retrovirus Particles

The preparation of infectious retroviral particles for cellular transduction was performed according to the "Stanford protocol" (Pear et al, 1993) in ΦNXgp (Phoenix) cells. Phoenix cells are a strain of HEK293T cells, which constitutively express the gag and pol genes of murine leukemia virus (MLV) and serve as packaging cells for infectious retrovirus particles. For each transfection via the calcium phosphate method Phoenix cells were plated in 10 cm dishes at a density of 2 x 10^6 cells per dish. The cells were transfected with 5 µg SF91-IRES-GFP-WPRE vector (Schwieger et al, 2002) encoding for the protein of interest, and vectors encoding for additional gag/pol proteins (10 µg) as well as env proteins (2 µg) conveying murine specificity (Beyer et al, 2002). To prepare transfection, the DNA was mixed with 61 µl 2M $CaCl_2$ and filled up to 500 µl with sterile MilliQ H_2O. Afterwards 500 µl of 2 x HBS solution was added drop wise while continuously "bubbling" into the solution by ejecting air through a stripette. The solution was incubated for 20 min at RT. The Phoenix cells were prepared by adding 25 µM (final concentration) chloroquine followed by the calcium phosphate solution, which was distributed evenly by careful agitation. The cells were incubated for 6 h at 37 °C and 5 % CO_2, until the medium was changed to 10 ml fresh complete medium (see 3.3.1) and the cells were left at 37 °C and 5% CO_2 for three to four days. During this incubation time the viral particles were released into the medium.

2 x HBS	273.8 mM NaCl, 0.296 mM HEPES, 21.6 mM Na_2POH_4 in MilliQ H_2O, pH 7.0

3.3.7 Retroviral Infection of MEFs

For retroviral infection MEFs were plated in 24-well multi-well dishes at 500 cells per well to provide 40 – 50% confluency. The infection medium was prepared by harvesting the virus-containing supernatant medium from transfected Phoenix cells (see 3.3.5) and passing it through a 0.8 µm filter to get rid of cell debris. Per transfection 500 µl of virus-containing supernatant was mixed with 500 µl fresh complete DMEM and supplemented with 0.1 mg/ml Polybrene. The infection medium was added to the cells and the plates were centrifuged for 1 h at 3000 rpm and 4 °C. After 3 h incubation at 37 °C and 5% CO_2, the medium was changed to fresh complete DMEM and the infected cells were cultured as described before (see 3.3.1). Leftover virus supernatant could be stored in aliquots at -80 °C to be used at later time points.

3.3.8 Flow Cytometry - FACS

For FACS analysis cells were grown in 6-well plates to 60 – 70% confluency, washed 1x with ice cold 1 x PBS, carefully scraped off the plates in 500 µl ice cold 1 x PBS with a cell scraper and centrifuged at 1200 rpm, 4 °C for 3 min. After washing once with 500 µl FACS buffer the cells were resuspended in 50 µl FACS buffer with or without fluorescence labeled antibody (diluted 1:200) and incubated for 15 min on ice in the dark. After washing twice with 500 µl FACS buffer the cells were resuspended in 300 µl FACS buffer and transferred to FACS tubes to be examined in a FACS Calibur.

FACS buffer	1 x PBS, 1 % FCS, 0.02 % sodium azide

3.3.9 Sorting Cells by Flow Cytometry

3 – 4 x 10^6 cells were prepared by washing once with sterile 1 x PBS at RT. Centrifugation of live cells was carried out at 900 rpm and RT for 3 min. The cell pellet was carefully resuspended in 500 µl 1 x PBS and passed through a cell sieve to avoid clumping of the cells. The cell suspension was kept in a sterile reaction tube on ice for no longer than 30 min until they were sorted in collaboration with Joachim Ellwart (Cell Sorting Service of the Institute of Molecular Immunology, HMGU) using a MoFlo cell sorter. Cells expressing the desired fluorescence protein (GFP or CFP) were collected and immediately plated onto 6-well multi-well dishes in 1 ml fresh complete DMEM medium to be cultured as described in 3.3.1.

3.3.10 Immunofluorescence

The exact location of a protein or other components inside a cell at a defined time point can be visualized by immunofluorescence. For this the cells were seeded at 30-40% confluence on microscopy cover slips. After adherence the cells were stimulated as needed and fixated with 2 ml PFA for 15 at RT. After washing with 1 x PBS the cells were permeabilized by incubating three times 5 min with 1.5 ml PBS/Triton, followed by three times 10 min incubation with 1.5 ml PBS+. The primary antibody against the protein of interest was diluted in 75 µl PBS+ and incubated overnight at 4 °C. After washing once with 1 x PBS the cells were again treated with 1.5 ml PBS/Triton for 10 min and with 1.5 ml PBS+ for 7 min. The secondary antibody was diluted 1:200 in 200 µl PBS+ and incubated 45 min at RT in the dark. Afterwards the cells were incubated again twice with 1.5 ml PBS+ for 5 min, washed with 1 x PBS and incubated with 2 ml DAPI-solution (1:10000 in 1 x PBS) for 90 sec. With the cells facing downwards the cover slips was placed on an 8 µl drop of 90% (v/v) glycerin in 1 x PBS on an object slide and sealed with

nail polish. The slides could be stored at 4 °C in the dark until the fluorescence was visualized with the help of a Leica TCS SP5 II confocal microscope.

PFA	2% (w/v) paraformaldehyde in 1 x PBS
PBS+	1% (w/v) BSA, 0.15% (w/v) glycine in 1 x PBS
PBS/Triton	0.15% v/v Triton X-100 in 1 x PBS

3.4 Protein Biochemistry

3.4.1 Total Protein Extraction

The required amount of cell lysis buffer was prepared by freshly adding protease- and phosphatase inhibitors into 0.1 % NP-40 lysis buffer. Adherent cells were quickly washed once with ice cold 1 x PBS, which was aspirated thoroughly, then the appropriate volume of lysis buffer was added directly onto the cells on the plate on ice. The cells were scraped off the plate with a cell scraper, resuspended and transferred to a fresh 1.5 ml test tube to be left on ice for 15 min. The cell lysate was cleared form insoluble and nuclear fragments by centrifugation (16,100 x g, 10 min, 4 °C) and used for experiments or kept at -20 °C for long time storage.

culture dish	lysis buffer
3 cm	50 µl
6 cm	100 µl
10 cm	200 µl
15 cm	400 µl

0.1% NP-40 lysis buffer	150 mM NaCl, 50 mM HEPES, 5 mM EDTA, 0.1 % (v/v) NP-40
inhibitors	0.5 mM sodium orthovanadate, 0.5 mM NaF, 0.5 mM sodium molybdate, 0.5 mM β-glycerophosphate, 0.5 mM sodium pyrophosphate, 1 mM phenylmethyl-sulfonyl fluoride, complete Mini protease inhibitor cocktail according to the manufacturer

3.4.2 Determining Protein Concentrations – Bradford Method

The Bradford solution was prepared freshly by mixing 1 volume of Bradford stock solution with 4 volumes of H_2O. 2 µl of cell lysate were thoroughly mixed into 800 µl of Bradford solution and incubated for 5 min at RT, before measuring the absorption at 595 nm and calculating the protein concentration with the help of the BioPhotometer and a BSA standard.

3.4.3 SDS-PAGE and Immunoblotting

Sodium dodecylsulfate polyacryamid gel electrophoresis (SDS-PAGE) was used to separate the proteins in a cell lysate according to their molecular weight. The gels were prepared by casting a 4 % stacking gel onto a 12.5 % separating gel between the glass plates of a Peqlab PerfectBlue Vertical Double Gel System. The desired amount of protein lysate was mixed with 1/6 volume of 6 x Laemmli sample buffer and heated to 95 °C for 5 min to denature the proteins. After loading the samples into the pockets of the stacking gel, the proteins were separated at 80 - 200 V.

12.5% gel	12.5% (w/v) polyacrylamide, 0.1% (w/v) SDS, 3.5 mM EDTA in 375 mM Tris-HCl pH 8.9
4% gel	4% (w/v) polyacrylamide, 0.1% (w/v) SDS, 3.5 mM EDTA in 125 mM Tris-HCL pH 6.8
SDS-PAGE running buffer	25 mM Tris, 192 mM glycine, 1% (w/v) SDS
6 x Laemmli buffer	150 mM Tris-HCl pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerin, 0.001% (w/v) bromphenolblue, 150 mM DTT

After separation the proteins were transferred onto nitrocellulose membranes by semi dry blotting with the Peqlab PerfectBlue Semi-dry Electroblotter for 2 h at a fixed current (mA = cm^2(blot) / 1.5). Afterwards the membranes were blocked for 1 h at RT in 5% (w/v) non-fat dry milk or BSA in 1 x TBSt, depending on the buffer used for the first primary antibody.

For immunoblotting the membranes were incubated in a 5% (w/v) milk or BSA solution with the primary antibody overnight at 4 °C. Generally, antibodies directed against phosphorylated proteins were incubated in BSA-solution, all others were incubate in milk. After washing three times with 1 x TBSt for 10 min at RT the membranes were incubated in 3% (w/v) non-fat dry milk with the appropriate HRP-coupled secondary antibody for 1 h at RT and subsequently washed again three times with 1 x TBSt. To visualize the protein bands each membrane was incubated for 1 min with 2 ml of an ECL solution, which was prepared by freshly adding 10 µl of ECL solution B into each 1 ml of ECL solution A. Luminescence signals were fixed onto x-ray films for different exposure times and developed with the help of an Optimax developing machine.

blotting buffer	120 mM glycine, 25 mM Tris
TBSt	10 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 0.1% (v/v) Tween-20
ECL solution A	200 mM p-coumaric acid, 1.25 mM Luminol, 100 mM Tris-HCl pH 8.8
ECL solution B	3% (v/v) H_2O_2

3.4.4 Luciferase Reporter Assay

The luciferase reporter assay is used to determine the activity of chosen transcription factors on a promoter, which is used to drive transcription of a luciferase protein. The relative luminescence of a given sample allows making assumptions about the activity of the respective signaling pathway in comparison to a control sample. To prepare for this, MEFs were transfected with expression-plasmids for HA-tagged LMP1 to serve as a signal inducer, along with plasmids encoding for an NF-κB driven luciferase reporter as well as a renilla-luciferase reporter gene to serve as a housekeeper for normalization. The cells were transfected by electroporation as described in 3.3.4 with 3 µg of the appropriate pCMV-HA-LMP1 plasmid, 3 µg 3xκB-Luc plasmid and 2 µg pPGK-Renilla plasmid. Each sample was additionally transfected with 3 µg peGFP plasmid to monitor the transfection efficiency. To reach the total amount of 20 µg for each transfection, salmon testes DNA was used to supplement the difference. After 24 h the cells were examined with the help of a fluorescence microscope to estimate the transfection efficiency by the amount of GFP expression. All reagents used for the luciferase reporter assay were obtained from the Promega Dual-Luciferase Reporter Assay System Kit and the protocol was followed accordingly. In short, the cells were washed once with ice cold 1 x PBS and lysed in 150 µl Luc-lysis buffer (Promega), scraped off the plates and transferred to fresh 1.5 ml Eppendorf tubes. After incubation at -80 °C for 2 – 24 h the lysate was cleared by centrifugation at 16,000 x g for 10 min at 4 °C. To measure the luciferase reporter activity 10 µl of each lysate was transferred into an opaque 96-well plate in duplicates, and the Luciferase Assay Buffer and Stop & Glo Buffer were prepared and used according to the manufacturer. The reaction was measured with the help of a lumimeter. To calculate the relative luminescence induction of each sample compared to a control, the duplicates of each luciferase value were averaged in a first step. Then the renilla values were normalized to the negative control, which was set to 1. Afterwards, each value obtained from the reporter gene was divided by the corresponding normalized renilla value to obtain the normalized RLU (relative luminescence unit) for each sample. These was again divided by the negative control to obtain the fold-induction.

3.4.5 Fractionation of Mouse Embryonic Fibroblasts for Lipid Raft Extraction

Isolation of the lipid raft fraction from cells is based on the fact that lipid rafts are insoluble in non-ionic detergents like Triton X-100 at 4 °C. Furthermore they are less dense than other cellular components, making it possible to isolate them inside a defined fraction within a discontinuous density gradient.

About 4 x 10^6 cells were cultured and stimulated as needed. The cells were washed once with ice cold 1 x PBS and scraped off the plate in 5 ml 1 x PBS to be transferred into a fresh 15 ml

Falcon tube on ice. After centrifugation for 5 min at 1000 rpm and 4 °C the cell pellet was homogenized in 450 µl ice cold TXNE buffer by resuspending 10 times with a 200 µl pipette tip. After 20 min incubation on ice the lysate was resuspended again as before and 380 µl was mixed with 520 µl of OptiPrep™ (corresponds to a density of 35% OptiPrep™). This lysate was transferred to the bottom of a 10 x 60 mm PA ultracentrifugation tube on ice and carefully covered with 2.5 ml 30% OptiPrep™ in TXNE without mixing the two phases, before carefully topping with 600 µl TXNE. Flotation of the rafts into the interphase between 0 and 30% density was achieved by ultracentrifugation at 160,000 x g for at least 4 h 20 min at 4 °C. Starting from the top of the tube 500 µl fractions were transferred into fresh 1.5 ml test tubes and prepared for analysis by SDS-PAGE. The rafts would be expected in fraction 2.

To confirm the isolation of the lipid rafts in fraction 2, 1 µl of each fraction was pipetted to a Nitrocellulose membrane. This dot blot was incubated overnight with HRP-coupled subunit B of cholera toxin (1:10000 in 5% milk in TBSt) to visualize the rafts with standard ECL (see 3.4.3).

TXNE	25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton X-100 TXNE buffer and OptiPrep™ were supplemented freshly with protease and phosphatase inhibitors as described in chapter 3.4.1

4 RESULTS

4.1 Establishment of an Experimental System for the Systematical Analysis of LMP1 Signaling

Studying LMP1 signaling with native wildtype LMP1 confronts researchers with a constitutively active molecule. This is insofar challenging, as signaling pathways commonly cross-regulate each other, which leads to different levels of regulation. This means that the activation of one pathway can determine the activation of another, secondary pathway, which does not originate at the primary receptor. These mechanisms of direct and indirect regulation cannot easily be discriminated in the case of a constitutively active receptor molecule like LMP1. Another challenge is the kinetics of pathway activity. One and the same pathway can be regulated by different mechanisms and possibly with different kinetics, and these, too, cannot be discerned when studying constitutive signaling. As described in chapter 1.6, certain aspects of LMP1 signaling still remain enigmatic or controversial. This is partly due to the above described fact that continuously active signaling is challenging to dissect, but also that a lot of data does not thoroughly discriminate between the CTAR domains of LMP1. Also, the precise role of TRAF and TRADD proteins in LMP1 signaling has not been systematically studied in comparable cellular backgrounds.

Therefore the need arose to study signaling of wildtype and mutated LMP1 in a comparable system in an inducible manner on the background of knockout cells derived from knockout mice.

4.1.1 A Genetically Clean Approach to Dissect LMP1 Signal Transduction: Knockout Mouse Embryonic Fibroblasts

To test the contribution of TRAF and TRADD proteins to LMP1 signaling in a comparable system, using wildtype and knockout mouse embryonic fibroblasts (MEFs) presented the most powerful tool. Although MEFs are not a natural target of EBV, the transforming potential of LMP1 was first described in fibroblasts (Wang et al, 1985), and many more studies concerning LMP1 signal transduction were conducted using fibroblast cell lines. Most importantly, a wide range of different knockout MEF cell lines is established and available, which is not true for other cell types. The present study was based on wildtype MEFs and MEFs deficient in TRADD (Ermolaeva et al, 2008), TRAF6 (Lomaga et al, 1999) or double-deficient in TRAF2 and 5 (Tada et al, 2001). These knockout cell lines were chosen, because previous studies had discussed the respective molecules as being involved in different signaling pathways induced by LMP1, as described in the introductory chapter 1.6. Additionally, the role of TRADD in LMP1 signaling had been established primarily based on overexpression studies. Only one single study was conducted in TRADD-deficient human B cells, which revealed a critical role for TRADD in the recruitment of IKK2 and the activation of the canonical NF-κB pathway by LMP1 in these cells (Schneider et al, 2008).

TRAF2/5 double-knockout cells were favored over single knockout cells, because it was shown that the lack of TRAF2 or TRAF5 alone was not sufficient to abolish TNFR-induced NF-κB activation (Nakano et al, 1999; Yeh et al, 1997). Cells lacking both TRAFs, however, were defective in their NF-κB response to TNFα-stimulation (Tada et al, 2001). Because of these partially redundant functions of TRAF2 and TRAF5, a double knockout cell line was chosen to rule out compensatory effects.

To verify that the obtained MEFs were lacking the designated molecules, and to test whether there were differences in the expression levels of the TRAFs and TRADD among the different cell lines, total cell lysates of all MEF cell lines were prepared and probed with antibodies against TRAF2, TRAF6 and TRADD in immunoblot experiments. The results showed clearly, that no TRADD, TRAF6 or TRAF2 proteins were present in the respective knockout cell lines (figure 4-1A). Furthermore, the results confirmed that the expression levels for TRADD, TRAF6 and TRAF2 were comparable among the different cell lines (figure 4-1A). It was not possible to verify the knockout of TRAF5 on the protein level, because no antibody was available that reliably detected endogenous TRAF5 in MEFs. Therefore the status of the cells was tested on

the genetic level by PCR from genomic DNA. The chosen approach was designed in accordance with the previously published data and the sequences for the primers used for the PCR were obtained from that study (figure 4-1B; Nakano et al, 1999). PCR performed with genomic DNA from wildtype MEFs generated a product of about 1 kb, which represents the wildtype sequence of the gene. A 1.2 kb fragment was amplified from the TRAF2/5$^{-/-}$ MEFs, which is representative of the replacement of exon 2 by a neo-cassette. At the same time, no PCR-product representing a wildtype allele could be amplified from the knockout cells. Hence, the results of the PCR verified that our batch of TRAF2/5$^{-/-}$ MEFs in fact carry a genetic knockout of the TRAF5 gene as published before (figure 4-1B and 4-1C).

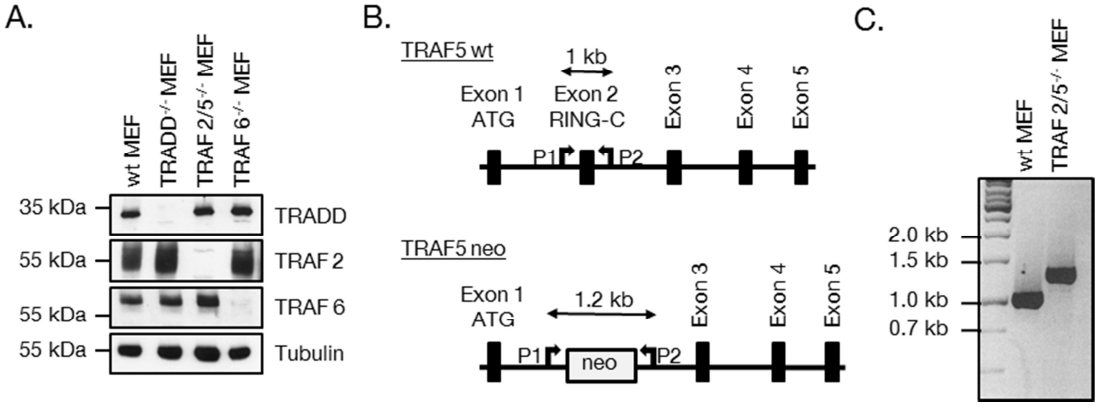

Figure 4-1. Verification of the knockout status of MEF cell lines by immunoblot and PCR. A. Total protein extracts of wildtype, TRADD$^{-/-}$, TRAF2/5$^{-/-}$ and TRAF6$^{-/-}$ MEFs were separated by SDS-PAGE and TRADD, TRAF2 and TRAF6 proteins were detected by immunoblotting. Tubulin served as a loading control. **B.** Schematic overview of the TRAF5 knockout strategy and expected PCR products for PCR with primers P1 and P2 (Nakano et al, 1999). **C.** Genomic DNA was extracted from wildtype and TRAF2/5$^{-/-}$ MEFs and subjected to PCR with Primers P1 and P2. The PCR products were separated on an agarose gel.

4.1.2 Analysis of NF-κB Signaling in Knockout MEFs by Transient Reporter Assays

It is well established that expression of LMP1 induces the activation of NF-κB (see introduction chapter 1.6.1). In order to gain a first impression of how the different knockout MEFs reacted to expression of LMP1 and LMP1 mutants, NF-κB luciferase reporter gene assays were performed. Wildtype and knockout cells were transfected transiently for 24 h with expression plasmids encoding HA-tagged LMP1wt or the indicated mutants (figure 4-2). To assess the contribution of the CTAR domains in combination with TRAF and TRADD molecules to NF-κB signaling, LMP1 constructs were used that lacked either a functional CTAR1 or CTAR2 domain. To disrupt signal

transduction originating from CTAR1 the TRAF-binding site P_{204}xQxT was mutated to A_{204}xAxA. As described in the introduction (chapter 1.2.1) this mutation blocks the binding of TRAF molecules to CTAR1, which is an important prerequisite for signal transduction (see chapter 1.6). The C-terminal amino acids P_{379}VQLSY within CTAR2 were identified to be essential for signal transduction originating at CTAR2 (see chapter 1.6.1). Therefore, deletion of the last 16 amino acids (Δ371) renders LMP1 incapable of triggering signaling at CTAR2 (Schneider et al, 2008). LMP1 disrupted in both CTAR domains (A_{204}xAxA/Δ371) was used as a negative control to evaluate the basal NF-κB levels of the cells. The used reporter plasmid contains three consecutive NF-κB responsive elements which induce transcription of a luciferase gene when activated (Mitchell & Sugden, 1995). The construct does, however, not discriminate among the NF-κB/Rel dimers and canonical or non-canonical NF-κB activity.

Since MEFs are not easy to transfect and the transfection efficiencies might vary, a plasmid encoding for green fluorescent protein (GFP) was co-transfected and the percentage of GFP-expressing cells was assessed by fluorescence microscopy prior to cell lysis. An average transfection efficiency of 30 – 50 % was achieved for the majority of experiments, although overall transfection was most efficient in wildtype and TRADD$^{-/-}$ MEFs, and slightly less efficient in TRAF6$^{-/-}$ and TRAF2/5$^{-/-}$ MEFs (data not shown).

In wildtype MEFs LMP1 induced NF-κB to an average of 9.1-fold compared to the signaling-dead mutant LMP1(A_{204}xAxA/Δ371). The overall induction dropped to about 6.5-fold when the CTAR1 mutant LMP1(A_{204}xAxA) was used, and was significantly reduced further to only 2.1-fold when CTAR2 was disrupted (figure 4-2, left panel). This result reflects prior observations that CTAR1 is required for about 30 % of total NF-κB activity in LMP1-transfected cells. CTAR2, on the other hand, was shown to contribute over 70 % of NF-κB activity, which is supported by the obtained data (Huen et al, 1995; Mitchell & Sugden, 1995).

Expression of LMP1wt in TRAF6$^{-/-}$ MEFs induced NF-κB-levels to an average of 5.6-fold (figure 4-2, middle left). A reduction compared to wildtype cells was expected, since TRAF6 was reported to be essential for the induction of canonical NF-κB by LMP1 (Schultheiss et al, 2001). Expression of the CTAR1 mutant LMP1(A_{204}xAxA) in TRAF6$^{-/-}$ MEFs led to NF-κB levels comparable to basal levels. This shows the essential role TRAF6 plays in signal transduction from CTAR2. Additionally, this result demonstrates that CTAR1 alone was responsible for the induction of NF-κB observed after the expression of LMP1wt or LMP1Δ371 in TRAF6-deficient cells, which is reflected by the comparable levels of NF-κB activity induced by LMP1wt and LMP1Δ371 in the TRAF6 knockout cells. Therefore, TRAF6 is not critically required to induce NF-κB activation originating at CTAR1. If at all, it might play a non-essential role.

Expression of LMP1wt or LMP1(A_{204}xAxA) induced NF-κB-activity to comparable levels in TRAF2/5$^{-/-}$ MEFs (figure 4-2, middle right). This result demonstrates that TRAF2 and/or TRAF5 are dispensable for NF-κB signal induction at CTAR2. The data further show that expression of the CTAR2 mutant in the TRAF2/5 double-knockout cells could not further induce NF-κB-activity compared to the CTAR1/2 mutant. This supports the fact that TRAF2/5 are essential for NF-κB activation from CTAR1.

LMP1wt induced NF-κB activity to about 4.2-fold in MEFs lacking TRADD (figure 4-2, right). This would attribute to only about half of the induction in wildtype cells, if wildtype and TRADD$^{-/-}$ MEFs are compared directly. Hence, TRADD might play a supporting, non-essential role in NF-κB activation in MEFs, which relates to previously published results that showed an involvement of TRADD in LMP1-induced NF-κB activation (Izumi & Kieff, 1997; Kieser et al, 1999; Schneider et al, 2008). Expression of LMP1(Δ371) significantly induced NF-κB levels to an average of 1.9-fold above basal levels. This effect of the CTAR2-mutation was comparable between wildtype and TRADD$^{-/-}$ MEFs, which demonstrates that TRADD is not needed for CTAR1-induced signaling.

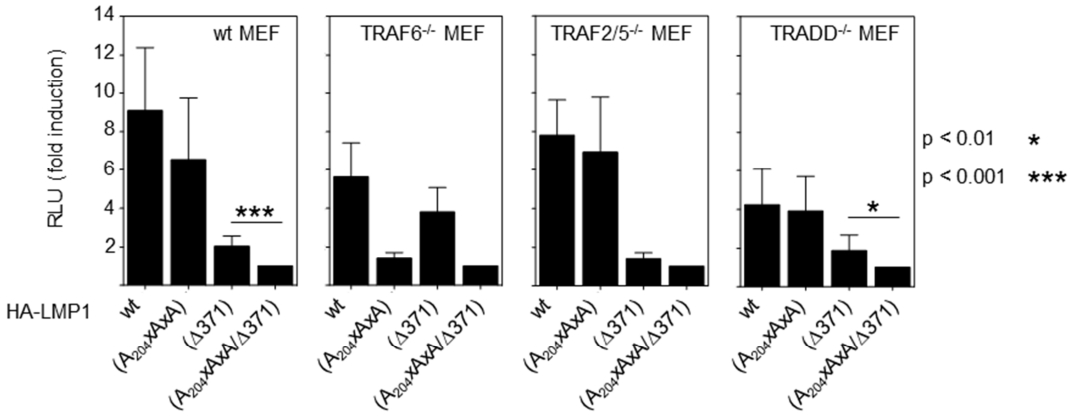

Figure 4-2. Luciferase reporter assays demonstrate the significance of TRAF and TRADD proteins in NFκB signaling. Wildtype and knockout MEFs were electroporated with expression plasmids for HA-LMP1wt, HA-LMP1(A_{204}xAxA), HA-LMP1(Δ371) and HA-LMP1(A_{204}xAxA/Δ371) along with the reporter plasmids 3xκB-Luc and pPGK-renilla as described in methods 3.4.4. A plasmid encoding for GFP was co-transfected as a control for the transfection efficiency. 24h post transfection the cells were lysed and firefly luciferase reporter assays with renilla under the control of a housekeeping gene promoter were performed as described in chapter 3.4.4. NF-κB luciferase activity was normalized to to the housekeeping reporter activity. HA-LMP1(A_{204}xAxA/Δ371) values were set to 1-fold induction. Data were generated by combining the results from 10 (wt and TRAF6$^{-/-}$), 9 (TRADD$^{-/-}$) and 5 (TRAF2/5$^{-/-}$) independent experiments. A non-paired heteroscedastic t-test was used to calculate the statistical significances as indicated. Error bars represent SD (standard deviation).

Taken together, the results from the NF-κB luciferase reporter assay showed that the cells responded to LMP1 expression by inducing NF-κB signaling as anticipated. This confirmed that the MEF system could be used for the comparable, systematical studies of LMP1 signaling. I showed that TRAF6 is critical for the induction of NF-κB at CTAR2, while CTAR1-dependent activation of NF-κB signaling relied essentially on the presence of TRAF2/5. TRADD, on the other hand, seems to play a non-essential, but supporting role in the induction of NF-κB signaling at CTAR2 in MEFs. It must be noted again that the luciferase reporter assay could not discriminate between canonical and non-canonical NF-κB. Yet, further elucidation of the signaling mechanisms was part of the main part of this study and was addressed with the help of inducible LMP1-receptors.

4.1.3 Retroviral Transduction of MEFs with Inducible NGF-Receptor-LMP1 Chimera

As mentioned before, it is challenging to study the direct molecular mechanisms and readouts of LMP1 signal induction with the native, constitutively active LMP1 molecule. Therefore, inducible LMP1 receptor-chimeras were expressed in MEFs. To this end a fusion protein consisting of the extracellular domain of the nerve growth factor receptor (NGFR) and the C-terminal, intracellular signaling domain of LMP1 was used (Gires et al, 1997) (Figure 4-3A). This chimera requires ligand-dependent oligomerization in order to trigger LMP1-dependent signaling pathways, which can be achieved by antibody crosslinking. In principle, the cells are labeled with antibodies directed against the extracellular domain of NGFR, which is then crosslinked by a secondary antibody for different periods of time to generate a time-dependent signaling response.

I aimed for the use of cell lines, which stably instead of transiently express the NGFR-LMP1 receptors, to achieve a high reproducibility. This is most effectively achieved by introducing the gene for the protein of interest to the cells by retroviral transduction, because this ensures the stable integration of the DNA of interest into the cellular genome. Therefore the sequences for NGFR-LMP1 were cloned into the retroviral vector SF91-IRES-GFP-WPRE (Schwieger et al, 2002). The NGFR-LMP1 constructs are expressed from the retroviral LTR (long terminal repeat) promoter. Further, the constructs contain a green fluorescent protein (GFP) reporter-gene, which is transcribed from the same bi-cistronic mRNA as the NGFR-LMP1 construct using an IRES (internal ribosomal entry site) cassette (figure 4-3B). This leads to the simultaneous expression of NGFR-LMP1 and GFP in the transduced cells from the same mRNA, and offered the possibility to sort for GFP in order to enrich NGFR-LMP1-positive cells. It also allowed fine tuning of the obtained bulk cell lines, because lower or higher GFP expression should coincide with lower or higher NGFR-LMP1 expression, respectively. The cartoon in figure 4-3B depicts the LTR-element containing NGFR-LMP1 and IRES-GFP sequences, and the way their stable

expression in MEFs can be exploited to trigger LMP1 signaling events in a time-dependent manner.

The obtained MEF:NGFR-LMP1 cells were supposed to be used to systematically study the impact, which the different CTAR domains of LMP1 have on signal transduction. Therefore, different NGFR-LMP1-mutants were cloned into the retroviral vector SF91-IRES-GFP-WPRE (Schwieger et al, 2002) (see material 2.4). In addition to the wildtype receptor NGFR-LMP1wt, three mutated NGFR-LMP1 receptors were cloned. To functionally disable the CTAR1 domain, the TRAF binding motif P_{204}xQxT was point-mutated to A_{204}xAxA (NGFR-LMP1(A_{204}xAxA)). A CTAR2-mutant was generated by replacing Y_{384} with a glycin residue (NGFR-LMP1(Y_{384}G)). This tyrosine residue at position 384 was found to be essential for CTAR2-induced signaling pathways (see introduction 1.6.1; Kieser et al, 1999). Both CTAR1 and CTAR2 were mutated accordingly to generate a double mutant (NGFR-LMP1(A_{204}xAxA/Y_{384}G)) (Figure 4-3C).

The NGF receptor (also called p75 neutrophin receptor (p75NTR)) is a member of the TNFR family and induces activation of NF-κB, MAPK pathways and PI3K/Akt (Chen et al, 2009). To make sure that the residual NGFR sequences of the constructs are in fact inactive, another negative control receptor was generated. This receptor was truncated after the transmembrane section (aa 1 – 280 of NGFR) and only comprised the extracellular NGFR domain (NGFRΔCD, Figure 4-3C, far right).

In order to transduce MEFs, retroviruses had to be generated that enclosed the DNA encoding for the target sequence. Therefore Phoenix (ΦNXgp) cells were transfected with the retroviral construct of interest and two further plasmids encoding for the retroviral proteins gag/pol and env, respectively, as described in the methods section (see 3.3.6). The viral envelope protein env was chosen to convey tropism for rodent cells, and the obtained retrovirus particles were used to infect MEFs as described in methods 3.3.7.

For a comprehensive, systematic evaluation of LMP1 signaling mechanisms, wildtype MEFs, TRAF6$^{-/-}$ MEFs, TRAF2/5$^{-/-}$ MEFs and TRADD$^{-/-}$ MEFs were transduced with the chimeras NGFR-LMP1wt, NGFR-LMP1(A_{204}xAxA), NGFR-LMP1(Y_{384}G) and NGFR-LMP1(A_{204}xAxA/Y_{384}G). This would result in a total of 16 cell lines. Additionally, the truncated NGFRΔCD receptor was introduced into wildtype MEFs.

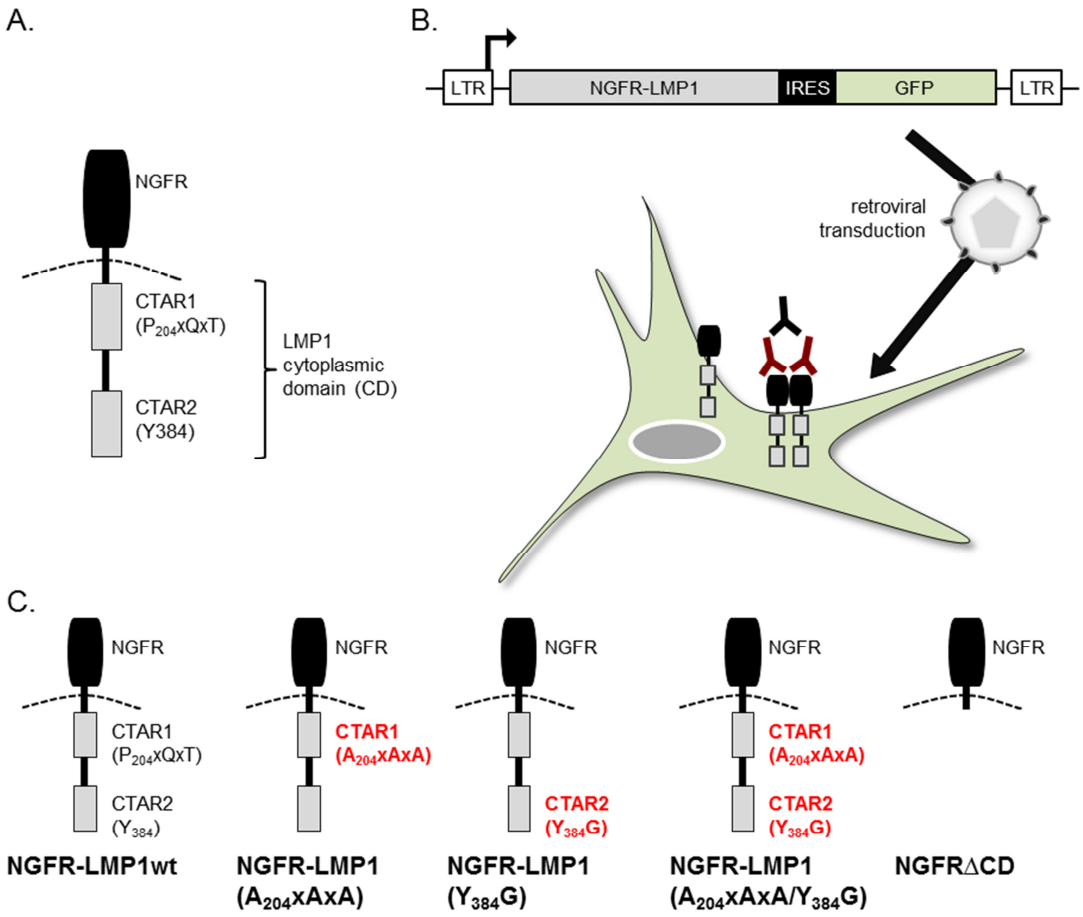

Figure 4-3. Schematic overview of the NGFR-LMP1 chimeric receptor and of the transduction strategy. A. The chimeric receptors consist of the NGF receptor consisting of the extracellular and transmembrane domains, which are fused to the intracellular signaling domain of LMP1. CTAR1 and CTAR2 are indicated. **B.** The sequence of the NGFR-LMP1 receptors was cloned into the retroviral expression vector SF91-IRES-GFP-WPRE. The NGFR-LMP1 sequence is followed by an IRES cassette, which enables the subsequent GFP gene to be translated from the same mRNA. After retroviral transduction MEFs stably express the NGRF-LMP1 receptors as well as GFP from the same mRNA. Antibody-dependent crosslinking of the NGFR-LMP1 receptors triggers LMP1 signaling events in a kinetic manner. **C.** Schematic overview of all chimeric NGFR-LMP1 receptors used in this study.

The aim of the study was to allocate different signaling pathways to specific LMP1 domains in combination with cellular signaling molecules. Therefore, the contribution of the CTAR domains to different signaling pathways would be studied in wildtype MEFs expressing NGFR-LMP1wt as well as the different CTAR mutants. To study the contribution of the signaling molecules, knockout MEFs expressing NGFR-LMP1wt would be employed. The resulting data would then be combined to learn the association of CTAR domains and signaling molecules with regard to their contribution to certain signaling pathways. In case a pathway turns out to be induced by

both CTAR domains, it would be necessary to include NGFR-LMP1 mutants in knockout backgrounds. Due to these combination possibilities, only a reduced set of cell lines were actually used for the initial experiments. The set included all four cell lines obtained from wildtype MEFs (wt MEF:NGFR-LMP1wt, wt MEF:NGFR-LMP1(A_{204}xAxA), wt MEF:NGFR-LMP1(Y_{384}G) and wt MEF:NGFR-LMP1(A_{204}xAxA/Y_{384}G), as well as each knockout cell line carrying the NGFR-LMP1wt receptor (TRAF6$^{-/-}$ MEF:NGFR-LMP1wt, TRAF2/5$^{-/-}$ MEF:NGFR-LMP1wt and TRADD$^{-/-}$MEF:NGFR-LMP1wt).

After infection with the respective virus supernatants the MEFs were expanded and sorted for GFP-positive cells by flow cytometry (see methods 3.3.9). This way a bulk cell population with great variation in GFP expression was obtained, instead of single cell clones. Hence no clonal variations were to be expected during the experiments. However, the first crosslinking experiments revealed that NGFR-LMP1wt constitutively induced phosphorylation of IκBα, regardless of antibody-crosslinking (data not shown). It was possible that a proportion of the cells expressed NGFR-LMP1wt at very high levels, so that the sheer overabundance of the receptors on the cell surface led to aggregation and with that to signal induction. Therefore the GFP-positive cell population was re-sorted for subpopulations containing cells with high, medium or low GFP expression (figure 4-4A). GFP expression in high and low GFP subpopulations was verified by flow cytometry and fluorescence microscopy (figure 4-4B and C). Flow cytometry revealed that the GFP levels were greatly reduced in GFP low cells (MFI = 311) compared to GFP high cells (MFI = 615). This resulted in a concomitant decrease of NGFR-LMP1 levels in the cells, and NGFR-LMP1 was reduced greatly in GFP low cells compared to GFP high cells (figure 4-4C).

The induction of the canonical NF-κB pathway was then tested in crosslinking experiments with both GFP low and GFP high cells (figure 4-4D). Constitutive phosphorylation of IκBα could be observed in unstimulated cells with high GFP expression, while no basal phosphorylation of IκBα was detected in the cells sorted for low GFP. Nonetheless, crosslinking of NGFR-LMP1wt successfully induced phosphorylation of IκBα in both cell lines after 20 to 30 minutes. The kinetics were similar in both cell lines, although the overall induction was not as high in GFP low cells, probably due to the lower basal level. Both cell populations could be stimulated with TNFα to a comparable extend, indicating that other cellular functions were not influenced by the expression of varying levels of GFP and/or NGFR-LMP1 (figure 4-4D).

These results showed that lower NGFR-LMP1 expression levels could be achieved by sorting the cells for low expression of GFP. It was possible to reduce background signals by lower NGFR-LMP1 expression. Nonetheless, signaling pathways were still inducible by NGFR-crosslinking.

All obtained MEF:NGFR-LMP1 cell lines were re-sorted for low expression of GFP. To keep the cell lines as comparable as possible, the sorting gate was not altered between different cell lines. Henceforth all studies were conducted with cell lines expressing low and comparable levels of NGFR-LMP1.

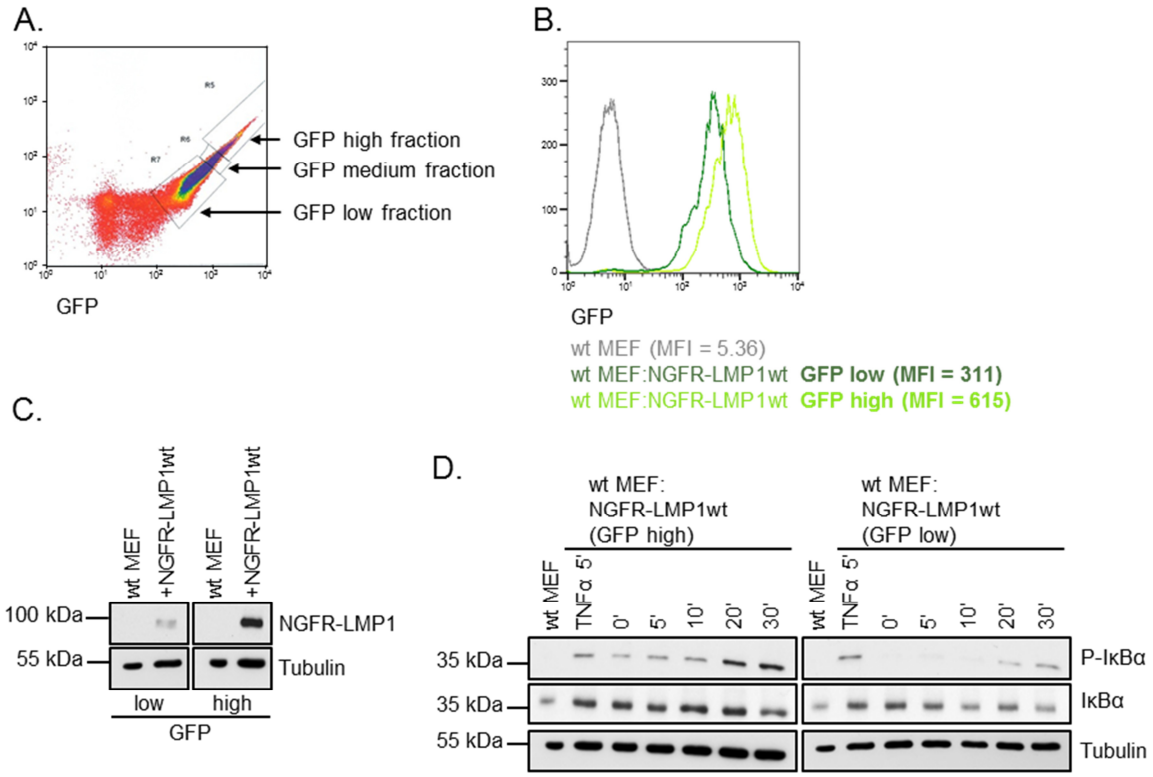

Figure 4-4. MEF:NGFR-LMP1 cells were sorted for low GFP-levels to avoid auto-accumulation of NGFR-LMP1 receptors by crowding on the cell surface. A-C. MEF:NGFR-LMP1 cells were sorted for GFP expression by FACS sorting. Different populations of cells expressing high, medium or low amounts of GFP were sorted to gain cell populations with high, medium and low amounts of NGFR-LMP1, respectively (**A.**). GFP high and low fractions were analyzed for GFP expression by flow cytometry (**B.**), and total protein levels of NGFR-LMP1 were analyzed by immunoblotting for LMP1 (**C.**). **D.** wt MEF:NGFR-LMP1wt cells sorted for high and low GFP expression were stimulated in a kinetic crosslinking experiment and examined for the response of the canonical NF-κB pathway. Cells were stained with 1 µg/ml α-NGFR for 1 h at 37°C, followed by different crosslinking periods with 10 µg/ml α-fc IgG/IgM at 37°C as indicated. As a control, cells were stimulated with 20 ng/ml TNFα for 5 min. Immunoblot analysis for phosphorylation at Ser32 and degradation of IκB display the induction of the canonical NF-κB pathway. Tubulin was used as a loading control.

4.1.4 Establishment of MEF Cell Lines Stably Expressing NGFR-LMP1 Receptors after Retroviral Transduction

Although the first experiments with wt MEF:NGFR-LMP1wt cells revealed that, in principle, it was possible to induce signaling by antibody-crosslinking of the receptors, the aforementioned set of obtained cell lines were tested again for the expression of NGFR-LMP1 by flow cytometry and immunoblot.

For effective crosslinking it is mandatory that the receptor is stably expressed on the cell surface. To examine surface expression of NGFR-LMP1, all cell lines were stained with a fluorescence-labeled antibody directed against NGFR (anti-NGFR A647) and evaluated by flow cytometry (figure 4-5). Additionally, to test whether crosslinking would induce changes in the surface expression of NGFR-LMP1, wt MEF:NGFR-LMP1wt cells were subjected to 1 h antibody crosslinking prior to flow cytometry.

First of all the results from flow cytometry clearly showed that no endogenous NGFR was expressed on wildtype MEFs that were not retrovirally transduced with NGFR-LMP1 (figure 4-5A). This was important to further rule out any unspecific signaling effects potentially resulting from antibody-crosslinking of endogenous p75NTR. All other transduced cell lines did express the NGFR-constructs on the cell surface. The expression of NGFR-LMP1wt was homogenous and comparable in wildtye MEFs and MEFs deficient for TRADD or TRAF6, as was the expression of NGFR-LMP1(Y_{384}G) (figure 4-5B, F, H and I). This ensured that signals induced by NGFR-LMP1 receptors are comparable.

Stimulation of the cells by antibody-crosslinking for 1 h resulted in a shift of the population towards cells expressing less NGFR-LMP1 on their surface, as could be seen after stimulation of wt MEF:NGFR-LMP1wt (figure 4-5D). The MFI dropped considerably compared to unstimulated cells and the population became less homogenous. This reflects internalization of the receptor after crosslinking and activation.

However, in some of the cell lines expression of NGFR-LMP1 on the surface was not homogenous (figure 5-4E, G and J). Both wt MEF:NGFR-LMP1(A_{204}xAxA) and TRAF2/5$^{-/-}$ MEF:NGFR-LMP1wt were distributed into two distinct subpopulations (figure 4-5E and J). One of these populations was comparable to the other cell lines, while the second population expressed NGFR-LMP1 about 100-fold less. The wildtype MEFs expressing the double mutant receptor NGFR-LMP1(A_{204}xAxA/Y_{384}G) made up an intermediate population that was more heterogeneous with regard to NGFR-LMP1 expression, which is reflected by the relatively low MFI (figure 4-5G).

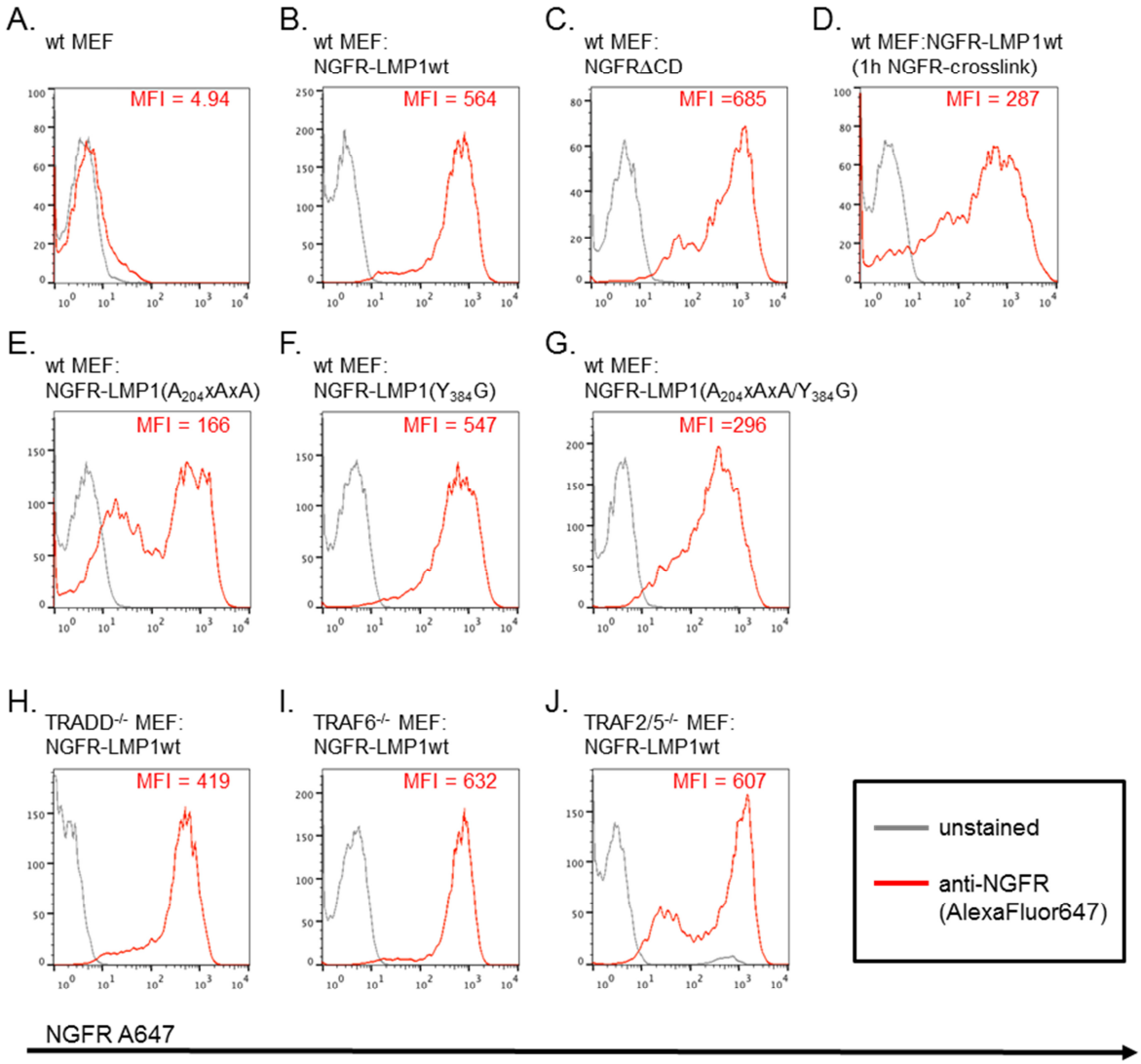

Figure 4-5. NGFR-LMP1 expression at the cell surface. NGFR-LMP1 was stained with antibodies against NGFR coupled to Alexa647 and analyzed with a FACS calibur flow cytometer using identical instrument settings for all samples. The cells were not permeabilized prior to staining in order to stain only surface expressed NGFR-LMP1. Each A647 stained sample was compared to an unstained control of the same cell line. FlowJo software was used to generate histogram plots and calculate mean fluorescence intensities. All cells had previously been sorted for low expression of GFP before according to figure 4-4A. One sample of wt MEF:NGFR-LMP1wt cells had been antibody-crosslinked before by labeling the cells with 1 µg/ml anti-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml IgG/IgM for 1 h at 37°C (panel D).

TRAFs 2 and 5 can bind to CTAR1 P_{204}xQxT, and CTAR1-dependent signal transduction has always been closely linked to TRAF2 associating with CTAR1. The fact that both the mutation of CTAR1 and the lack of TRAF2/5 leads to the appearance of a second population of cells with less NGFR-LMP1 raises the assumption that the expression of NGFR-LMP1 in MEFs is influenced by the integrity of CTAR1 and its ability to induce signaling. Even though NGFR-

LMP1 should not induce signaling in the absence of a stimulus, it is possible that the expression of NGFR-LMP1 leads to a certain selection process within the cell population for cells with lower expression of NGFR-LMP1, when CTAR1 is dysfunctional. Unknown “tonic” signaling events at CTAR1 could counter this selection process. It could also mean that NGFR-LMP1 with a mutated CTAR1 domain or a flawed CTAR1 signaling complex is inefficiently expressed on the cell surface.

It was only possible to analyze the presence of the extracellular proportion of the NGFR-LMP1 receptors using flow cytometry with unpermeabilized cells. To assess the expression of the complete receptor constructs including the intracellular signaling domain of LMP1 in the transduced cells, total cell lysates were generated, separated by gel electrophoresis and probed with antibodies directed against the signaling domain of LMP1 (Figure 4-6).

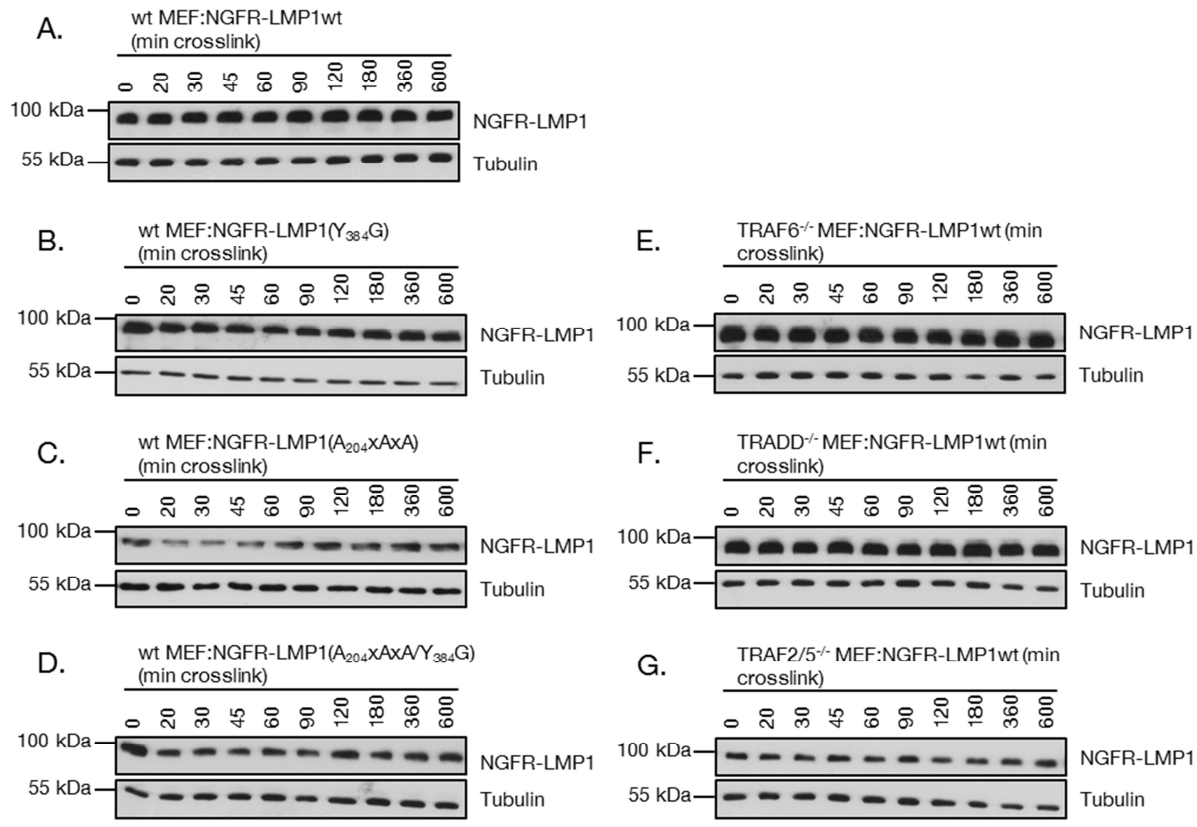

Figure 4-6. NGFR-LMP1 protein levels in NGFR-LMP1 MEFs after crosslinking. The indicated cell lines were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C followed by 10 µg/ml α-fc IgG/IgM at 37°C for the indicated times between 20 min and 10 h. Expression of NGFR-LMP1 was visualized by immunoblotting using an antibody directed against the LMP1 signaling domain (α-LMP1 3G6-1). Tubulin served as a loading control. Protein levels of panels A – G are directly comparable.

Expression of NGFR-LMP1 was observed in all samples. Even over a long stimulation with crosslinking-antibodies the protein levels of LMP1 remained more or less stable. However, the differences in surface expression of NGFR-LMP1 that could be observed in the flow cytometry assay were mirrored by the immunoblot results. Overall expression of NGFR-LMP1 is weakest in TRA2/5$^{-/-}$ MEFs and in cells expressing the receptor with a mutated CTAR1 domain ((A_{204}xAxA/) and (A_{204}xAxA/Y_{384}G)) (figure 4-6C, D, G). The total protein levels for NGFR-LMP1wt in TRAF6$^{-/-}$ MEFs (figure 4-6E) were slightly higher than those for NGFR-LMP1wt in wildtype MEFs (figure 4-6A), which is in accordance with the FACS data (figure 4-5A and I).

4.1.5 Antibody-Crosslinking Induces Oligomerization of NGFR-LMP1 on the Cell Surface

LMP1 needs to form clusters in the membrane in order to induce signaling. As discussed before, native LMP1 aggregates in the absence of a ligand via its transmembrane domains (see introduction 1.2.1). The NGFR-LMP1 system is based on the principle that signaling is triggered in response to antibody-mediated crosslinking of the receptors. To verify that the NGFR-LMP1 receptors in fact aggregated through crosslinking, immunofluorescence staining of NGFR-LMP1 in unstimulated and stimulated wt MEF:NGFR-LMP1wt cells was performed. The cells were fixed and permeabilized after 0, 30 or 90 minutes of crosslinking and stained with anti-LMP1 antibodies and secondary antibodies coupled to Cy3 fluorochrome to visualize NGFR-LMP1. DAPI was used to visualize the nucleus of the cells and GFP was observable as a result of viral transduction.

Confocal microscopy revealed that NGFR-LMP1 was evenly distributed in unstimulated cells (figure 4-7). This changed rapidly upon antibody crosslinking. Already 30 minutes after stimulation NGFR-LMP1 aggregated in patches within the cell membrane. These accumulations were still present after 90 minutes of crosslinking, although larger patches were now visible inside the cell, which reflects internalization of NGFR-LMP1. This result confirms the flow cytometry data, where a reduction of NGFR-LMP1 on the cell surface was observed after crosslinking stimulation (figure4-5D).

These results demonstrate that antibody-crosslinking rapidly induces the oligomerization of NGFR-LMP1 on the cell surface, which is a prerequisite for the triggering of signal-transduction.

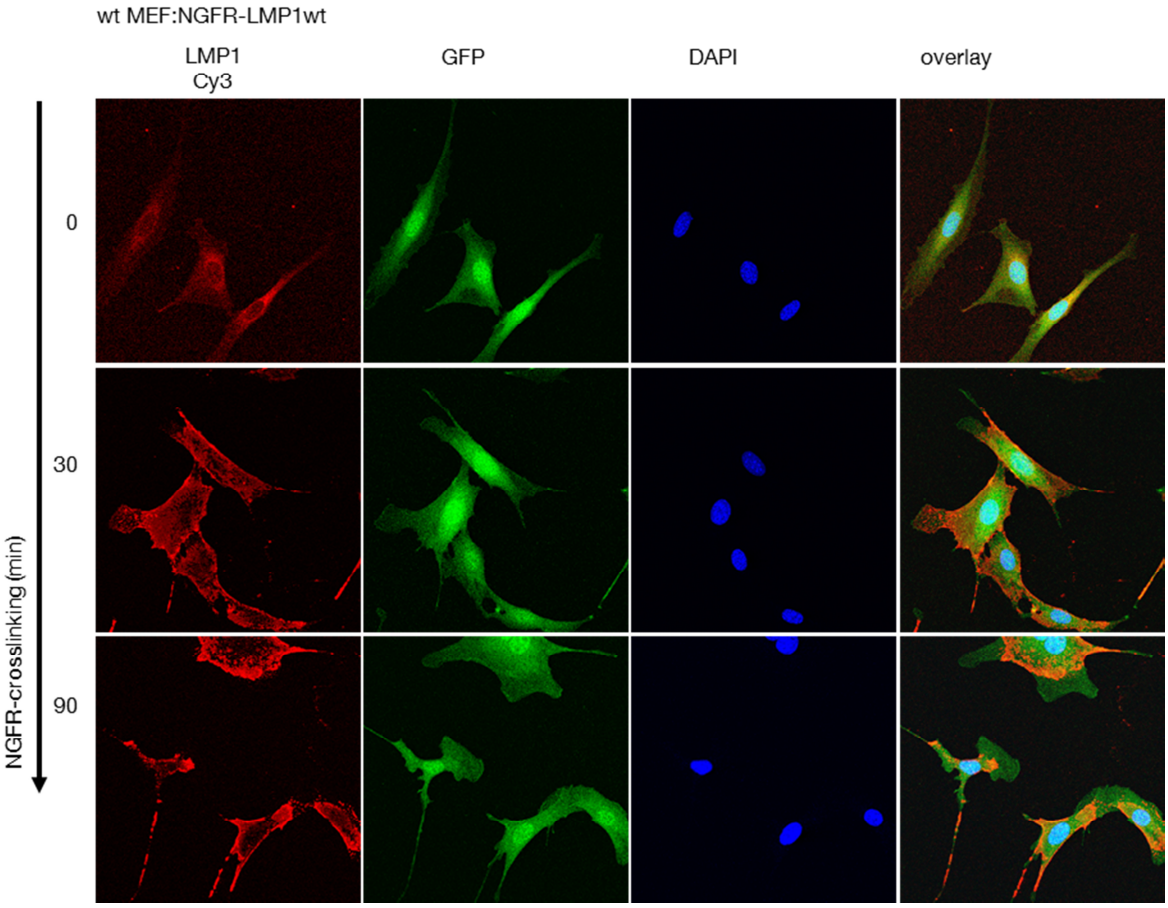

Figure 4-7. Immunofluorescence staining of LMP1 reveals its clustering in the membrane after NGFR-crosslinking. wt MEF:NGFR-LMP1wt cells were incubated with 1 µg/ml α-NGFR antibody for 1 h at 37°C and stimulated with 10 µg/ml α-fc IgG/IgM for 30 and 90 min. After fixation with PFA the cells were stained with α-LMP1 (CS1-4) and a secondary antibody coupled to Cy3 as well as DAPI and analyzed with the help of a Leica TCS SP5 II confocal microscope (63 x optical magnification).

4.1.6 The Truncated NGFRΔCD Receptor Does Not Induce Signaling in MEFs

It was mandatory to ensure that crosslinking of NGFR-LMP1 by antibody-crosslinking did not induce unspecific signaling. To test if crosslinking would induce putative assembly and activation of the extracellular NGFR-domain and other receptors like Trk receptor kinase, which is a neutropin receptor that can form dimers with p75 (Chao & Hempstead, 1995), a truncated receptor molecule (NGFRΔCD) was expressed in wildtype MEFs (figure4-3C far right). It consisted solely of the extracellular and transmembrane domain of NGFR without the intracellular signaling domain of LMP1. To compare signaling activation of NGFR-LMP1wt and NGFRΔCD, wildtype MEFs expressing either receptor were subjected to antibody-crosslinking for up to 60 minutes.

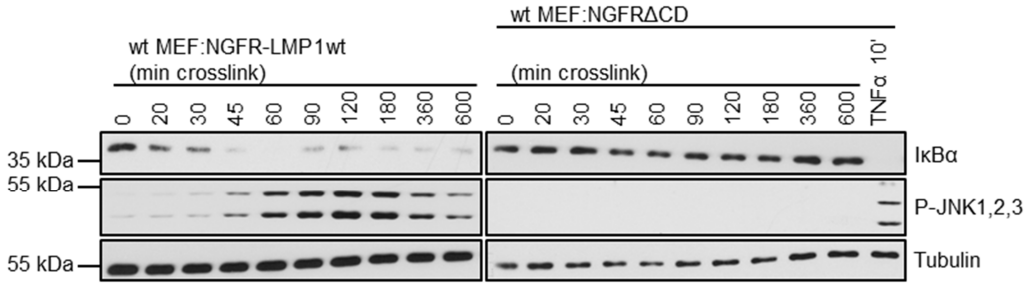

Figure 4-8. The NGFR extracellular part of the NGFR-LMP1 construct does not unspecifically induce signaling in MEFs upon crosslinking. Widltype MEFs stably expressing NGFR-LMP1wt and NGFRΔCD were subjected to antibody-crosslinking with 1 µg/ml α-NGFR for 1 h at 37°C and 10 µg/ml α-fc IgG/IgM at 37°C for the indicated time points. Stimulation with 20 ng/ml TNFα served as a positive signaling control. Induction of signaling pathways was analyzed by immunoblotting for different signaling molecules as indicated. The phosphorylation sites in P-JNK assayed were Thr183/Tyr185. Tubulin served as a loading control.

As can be concluded from figure 4-8, NGFR-LMP1wt clearly induced the canonical NF-κB pathway and the JNK pathway, which can be seen from the degradation of IκBα and the phosphorylation of JNK, respectively. Both pathways were induced between 30 and 45 minutes of crosslinking. No degradation of IκBα and no phosphorylation of JNK were observed upon crosslinking of NGFRΔCD, indicating that the extracellular and transmembrane NGFR-fragment alone is not sufficient to induce these signaling pathways. As a control, TNFα stimulation of wt MEF:NGFRΔCD cells did result in the activation of both pathways.

Taken together the results from the stimulation experiment showed that NGFR-crosslinking induces activation of signaling pathways in an LMP1-specific manner. Similarly, no unspecific signal induction was observed when wildtype MEFs, which were not transduced with any NGFR construct, were stimulated by anti-NGFR crosslinking (data not shown).

Notably, signal induction by NGFR-crosslinking started only after 20 to 30 minutes. This is rather later compared to the TNFα control, which induced IκBα degradation and phosphorylation of JNK after only 5 to 10 minutes. Therefore it is possible that signal transduction by NGFR-LMP1 crosslinking is generally slower than the induction of signaling pathways by ligand-dependent activation of cellular receptors like TNFR.

4.1.7 Stimulation of Cells with TNFα and IGF-1

LMP1 mimics the constitutively active TNF receptor family member CD40 (see introduction 1.2) and collectively induces several signaling pathways that are associated with numerous cellular receptors. To control the responsiveness of the MEF:NGFR-LMP1 cells to cellular signaling pathways, all cells were stimulated with TNFα and IGF-1 as a control. TNFα is a known inducer of the canonical NF-κB and JNK pathways, and both TRADD and TRAF2/5 are essential for this (see introduction 1.5). IGF-1 on the other hand potently activates the PI3K/Akt pathway, and there have been reports that TRAF6 can be involved in activation of Akt (Wang et al, 2006; Yang et al, 2009b).

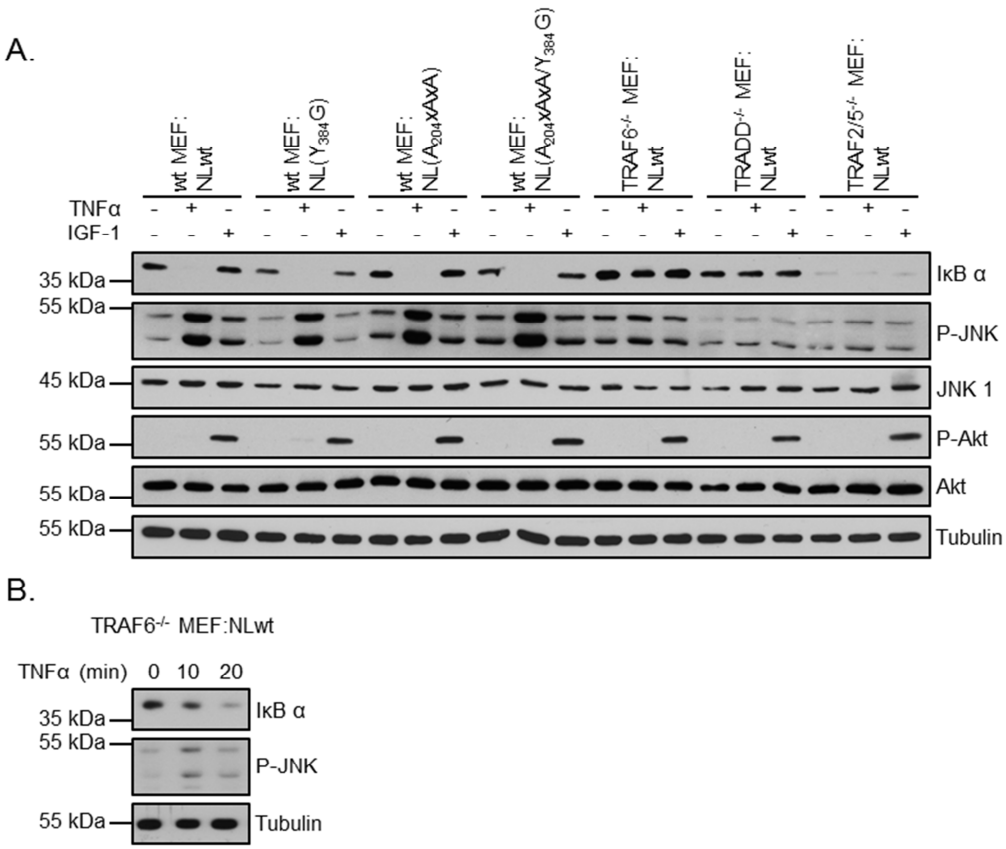

Figure 4-9. Response of MEF:NGFR-LMP1 cells to TNFα and IGF-1 stimulation. A. Wildtype and knockout MEFs stably expressing NGFR-LMP1wt and mutant receptors as indicated were stimulated with either 20 ng/ml TNFα or 20 ng/ml IGF-1 for 10 minutes. Signaling response to these stimuli was assessed by immunoblotting for IκBα, JNK phosphorylated at Thr182 and Tyr184 or Akt with Ser473 phosphorylation. Tubulin served as a loading control. **B.** TRAF6$^{-/-}$ MEF:NGFR-LMP1wt cells were stimulated with 20 ng/ml TNFα for 10 and 20 minutes. Immunoblots were performed as indicated and as described above.

The results in figure 4-9A show that stimulation with IGF-1 resulted in rapid phosphorylation of Akt in all cell lines, regardless of NGFR-LMP1 receptors or knockout status. Also, activation of Akt was comparable in TRAF6$^{-/-}$ MEFs and wildtype MEFs, which appeared contradictory to the results published by Yang et al. (Yang et al, 2009b). However, the data by Yang et al. also show residual Ser473 phosphorylation in TRAF6$^{-/-}$ MEFs upon IGF-1 treatment, and their Akt activation kinetics was much slower, occurring only after 15 – 30 minutes of IGF-1 stimulation. Yang et al. did not specify the amount of IGF-1 used in their experiments, so it is well possible that the effects of the TRAF6 knockout are only visible with lower concentrations of IGF-1. Furthermore, overexpression of TRAF6 enhanced only Thr308-, but not Ser473-phosphorylation of Akt, which argues for a selectivity of TRAF6-contribution towards Thr308-phosphorylation (Yang et al, 2009b).

TNFα-treatment induced degradation of IκBα in all wildtype MEFs, but not in TRADD$^{-/-}$ and TRAF2/5$^{-/-}$ MEFs (figure 4-9A). This was expected and indicates that these cell lines respond normally to TNFα, with regard to the canonical NF-κB pathway. However, IκBα levels were much lower in TRAF2/5$^{-/-}$ MEFs compared to wildtype MEFs. In fact, it is known that the lack of TRAF2 results in deregulated, constitutive activation of the canonical NF-κB pathway due to elevated basal IKK activity, which leads to the degradation of IκBα (Zhang et al, 2009). Notably, IκBα degradation was delayed in TRAF6$^{-/-}$ MEFs and was only visible after 20 minutes of TNFα treatment (figure 4-9B). TNFα also potently induced activation of the JNK pathway in wildtype MEFs. As expected, lack of TRADD or TRAF2/5 completely abolished JNK phosphorylation through stimulation with TNFα. In TRAF6$^{-/-}$ MEFs JNK was phosphorylated after TNFα treatment, but this phosphorylation was much weaker compared to wildtype MEFs (figure 4-9A and B).

The results show that it was possible to induce signaling responses to external stimuli in all cell lines. Wildtype MEFs and MEFs deficient in TRADD or TRAF2/5 respond as expected to TNFα and IGF-1. However, the response to TNFα in TRAF6$^{-/-}$ MEFs was slightly delayed regarding IκBα degradation and overall weaker regarding JNK phosphorylation.

4.2 Systematical, Comprehensive Analysis of LMP1-Induced Signaling Pathways

The primary objective of this work was to establish a system of inducible NGFR-LMP1 receptors in wildtype and knockout MEFs in order to perform systematical analysis of LMP1-induced signaling pathways.

Since one of the advantages of an inducible receptor is that the kinetics of signaling can be studied in detail, all experiments were conducted in long term kinetics with different stimulation steps lasting from 20 minutes up to 10 hours. The long term kinetics also enabled the discrimination between direct and indirect signaling pathways. Direct signaling occurs within minutes after the initial stimulus and is independent of gene transcription.

When doing a systematical screening, the utmost comparability among the obtained results is mandatory. However, as described before, the lack of TRAF2 results in deregulation of the non-canonical and the canonical NF-κB pathway, and p100 as well as IκBα are constitutively degraded in cells lacking TRAF2 (Grech et al, 2004; Song & Kang, 2010; Vallabhapurapu et al, 2008; Zhang et al, 2009). Of course, this can have a great impact on the intracellular environment, and with that it can greatly influence the responsiveness of these cells to external stimuli, even if TRAF2 itself might not be involved in a particular pathway. As shown before, IκBα levels were lower in TRAF2/5$^{-/-}$ MEFs compared with the other MEF cell lines (figure 4-9A), which is likely due to the inhibitory effect TRAF2 elicits on the canonical NF-κB pathway (Zhang et al, 2009). It was also clear from the results obtained by flow cytometry that expression of NGFR-LMP1 with mutations in the CTAR1 domain, but also expression of NGFR-LMP1wt in TRAF2/5$^{-/-}$ MEFs differed substantially from all other cell lines (see 4.1.4). Therefore it was obvious that results gained from experiments with TRAF2/5$^{-/-}$ MEFs and wildtype MEFs expressing NGFR-LMP1(A_{204}xAxA) would not be comparable with the other cell lines, especially not in a quantitative way. This led to an exclusion of both cell lines from the initial systematical analysis.

4.2.1 Induction of the Canonical NF-κB Pathway

Although extensive research in the past years has unraveled many aspects of how LMP1 induces the canonical NF-κB pathway, some aspects still remain unclear or controversial. By using a comprehensive system of inducible LMP1 receptors, I wanted to shed more light on the kinetics and mechanisms of activation of canonical NF-κB by LMP1. One of the critical steps in activation of the canonical NF-κB pathway is the degradation of IκB. As described in the introductory chapter 1.5.1, degradation of IκB is necessary to liberate NF-κB dimers and thereby enable them to translocate to the nucleus to regulate gene transcription. Therefore all immunoblots were probed with antibodies against total IκBα, to monitor the inducible decrease of the protein over time.

IκBα was readily degraded by stimulation of NGFR-LMP1wt in wildtype MEFs (figure 4-10A). This degradation started as early as 30 minutes after addition of IgG/IgM crosslinking antibodies to the cells. Levels of IκBα remained low until around 180 minutes, when a gradual increase in

protein levels commenced. It is known that the canonical NF-κB pathway terminates itself by upregulating the transcription of new IκBα molecules, and that IκBα itself is a target of NF-κB signaling by LMP1 (Gewurz et al, 2012).

Mutation of the CTAR2 domain to $Y_{384}G$ or the lack of TRAF6 also completely abolished degradation of IκBα upon NGFR-crosslinking (figure 4-10B and D). This shows that CTAR2 alone is responsible for the induction of the canonical NF-κB pathway, and that this critically relies on TRAF6.

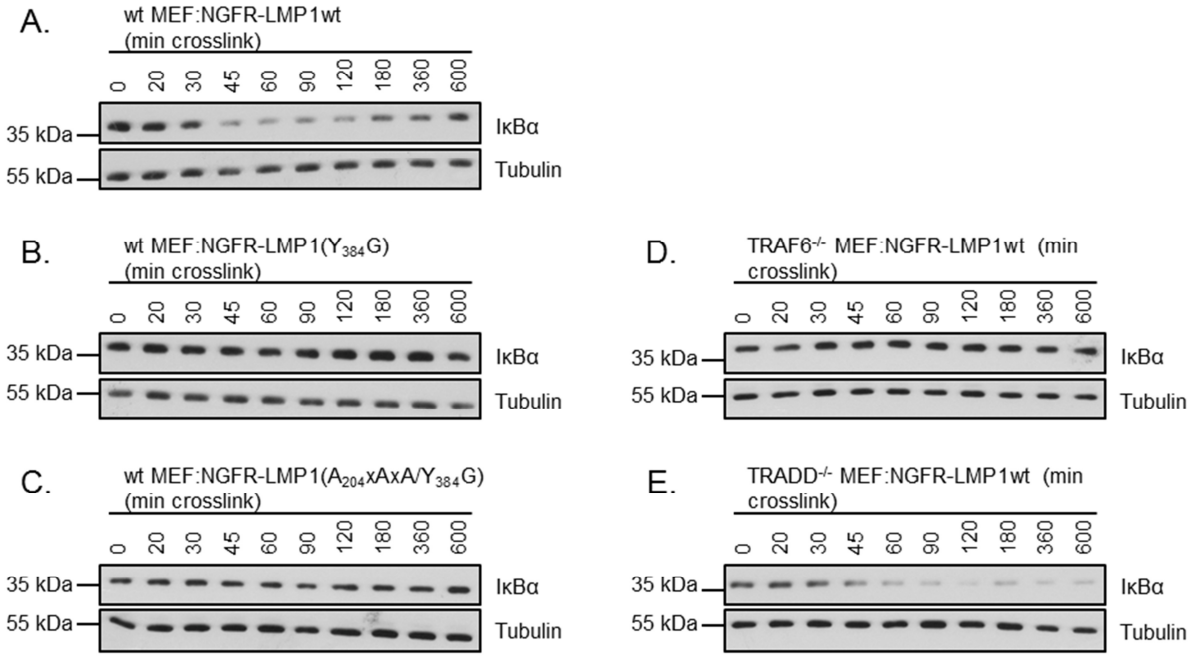

Figure 4-10. Analysis of the canonical NF-κB pathway induced by NGFR-LMP1 receptors. Wildtye and knockout MEFs stably expressing NGFR-LMP1wt and mutant receptors as indicated were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml α-fc IgG/IgM at 37°C for the indicated time points between 20 min and 10 h. The induction of the canonical NF-κB pathway was visualized by immunoblotting for the degradation of IκBα. Tubulin served as a loading control. Each knockout or mutant was directly compared to a wt MEF:NGFR-LMP1wt control on the same blotting membrane, and each blot shown here was chosen in a way to represent comparable results with regard to the wildtype control.

As expected, mutation of both CTAR domains abrogated degradation of IκBα upon crosslinking (figure 4-10C). This also confirms again that no unspecific signaling effects are triggered by antibody-stimulation.

Schneider et al. suggested that TRADD is a critical mediator of the LMP1-induced canonical NF-κB pathway in B cells by recruiting IKKβ (Schneider et al, 2008). Inducible NGFR-LMP1,

however, does not critically rely on TRADD to initiate the degradation of IκBα in MEFs (figure 4-10E). In comparison with wildtype cells, the onset of degradation is slightly delayed from 30 minutes in wildtype MEFs to 45 minutes in TRADD$^{-/-}$ MEFs. Therefore it is possible that TRADD contributes to the activation of canonical NF-κB, but does not play an essential role. Figure 4-10E further shows that the re-expression of IκBα in TRADD$^{-/-}$ MEFs at later time points does not occur and no increase of IκBα was observed after 180 minutes. This could point to two conclusions. On the one hand, the lack of TRADD could lead to a hyperactivation of the canonical NF-κB pathway, and newly synthesized IκBα molecules are constantly degraded. Another possibility could be that with the lack of TRADD LMP1 signaling fails to sufficiently induce transcription of IκBα.

Taken together, stimulation of NGFR-LMP1 in MEFs offers a powerful tool to compare the impact of the mutation of CTAR2 or the lack of TRAF6 or TRADD on LMP1-dependent induction of the canonical NF-κB pathway. The results demonstrate that IκBα degradation starts early after stimulation of NGFR-LMP1wt, and that this can be completely blocked by mutation of CTAR2 or the lack of TRAF6. The role of TRADD in activating the canonical NF-κB pathway in MEFs seems to be non-essential. It is, however, possible that there is a difference between inducible and constitutive LMP1-dependent activation of NF-κB, with regard to the role of TRADD. As could be seen in the luciferase reporter assays (chapter 4.1.2), TRADD played a supporting role in the induction of NF-κB, whereas the degradation of IκBα in response to NGFR-LMP1 crosslinking was largely unaffected.

4.2.2 Induction of MAPK Pathways – the JNK Pathway

In order to assess the induction of the JNK pathway, immunoblots were probed with antibodies directed against phosphorylated JNK. Three isoforms of JNK exist (JNK1, JNK2 and JNK3), which are phosphorylated on threonine and tyrosine residues (Thr183 and Tyr185) upon activation.

As can be seen in figure 4-11A the JNK pathway was activated by phosphorylation of JNK after approximately 30 to 45 minutes of crosslinking-stimulation in wt MEF:NGFR-LMP1wt. Maximum phosphorylation was achieved after 60 minutes and declined after 180 minutes. This kinetics is similar to the degradation of IκBα (figure 4-10A), which suggests that both the canonical NF-κB and the JNK pathway are initialized at a shared time point. This supports the fact that both pathways are initiated by the same complex around TNIK, as published before (Shkoda et al, 2012).

Again, simultaneous mutation of both CTAR1 and CTAR2 abolished the induction of the JNK pathway, serving as a negative control for unspecific background signals (figure 4-11C).

The induction of the JNK pathway was previously described to be originating at CTAR2 (Eliopoulos & Young, 1998; Kieser et al, 1997). This result was confirmed with wt MEF:NGFR-LMP1($Y_{384}G$), which failed to induce JNK phosphorylation upon antibody-crosslinking (figure 4-11B). The same was true for TRAF6-deficient cells, where no activation of JNK occurred at any time point of stimulation (figure 4-11D). Taken together these results show that induction of the JNK pathway critically relies on a functional CTAR2 domain, and that TRAF6 plays an essential role in this.

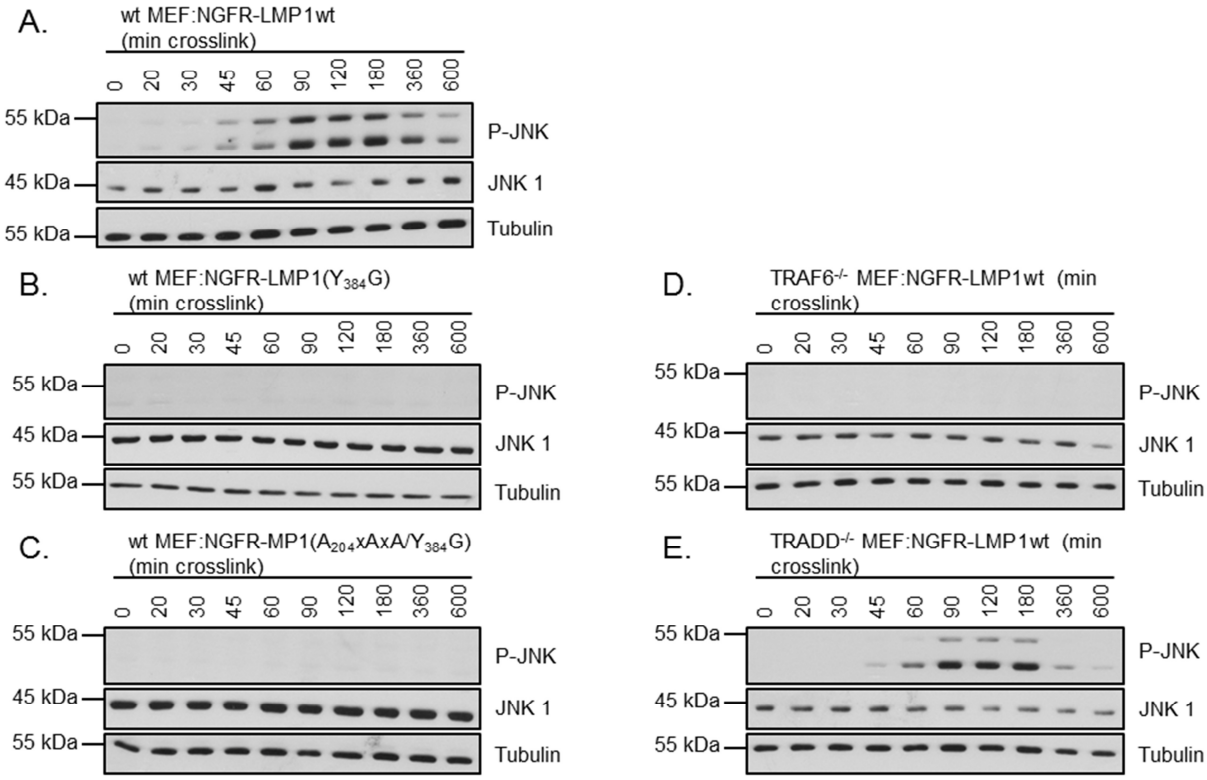

Figure 4-11. Analysis of the JNK pathway induced by NGFR-LMP1 receptors. Wildtype and knockout MEFs stably expressing NGFR-LMP1wt and mutant receptors as indicated were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml α-fc IgG/IgM at 37°C in a kinetic manner for the indicated time points between 20 min and 10 h. Induction of the JNK pathway was assayed by immunoblotting for phosphorylation of JNK at Thr183 and Tyr185. Tubulin served as a loading control.

The involvement of TRADD in JNK signaling has been discussed controversially in the past. As described in the introduction, the role of TRADD in JNK activation is not entirely clear, and there have been reports that ascribe a contributing role to TRADD (Eliopoulos et al, 1999a), while others have demonstrated that the lack of TRADD does not interfere with JNK activation by

LMP1 (Schneider et al, 2008; Wan et al, 2004). Clearly, phosphorylation of JNK upon NGFR-LMP1wt crosslinking is induced in TRADD$^{-/-}$ MEFs, but the overall induction is much weaker than in wildtype cells, although the kinetics appears similar in both cell lines (figure 4-11E). The signal is terminated after 180 minutes of crosslinking, which is the same in wildtype MEFs. This points to a supporting role for TRADD in JNK activation by LMP1 in these cells, but it is not essential for the induction of the JNK pathway.

Taken together, the complete lack of JNK activation in cells lacking TRAF6 or upon crosslinking of a receptor with a defective CTAR2 domain shows that CTAR2 and TRAF6 are critical mediators of JNK activation in MEFs, and that no activation of JNK occurs via CTAR1 in the absence of a functional CTAR2 domain. Therefore, CTAR1 is not involved in the activation of JNK by LMP1 in MEFs. At the same time, TRADD plays a minor, contributing role in the activation of JNK by NGFR-LMP1 in MEFs.

4.2.3 Activation of the p38/MAPK Pathway

The kinase p38 is the MAP3K of the p38/MAPK pathway and must be phosphorylated in order to induce specific transcription. Therefore, the induction of the p38/MAPK pathway by LMP1 was assessed by using antibodies for immunoblotting, which are directed against p38 phosphorylated at Tyr180 and Tyr182.

Phosphorylation of p38 was induced as early as 45 minutes after crosslinking of NGFR-LMP1wt receptor in wildtype MEFs (figure 4-12A). A peak was reached after 90 to 120 minutes and the signal was terminated again after 360 minutes. This kinetics generally coincides with the induction of the JNK pathway and the degradation of IκBα, again showing that all three pathways are likely induced at around the same early time point in LMP1 signal transduction.

NGFR-LMP1wt failed to induce the p38/MAPK pathway upon stimulation in cells lacking TRAF6 (figure 4-12D). This confirms previous results, which attributed a critical role to TRAF6 in activating p38/MAPK (Schultheiss et al, 2001). A role for TRADD was so far only shown by using dominant negative TRADD lacking the death domain, which interfered with p38 activation by LMP1 (Schultheiss et al, 2001). In fact, lack of TRADD reduced the phosphorylation of p38 upon antibody-crosslinking, but did not fully abolish it (figure 4-12E). These data demonstrate that the TRADD protein does play a supportive role in the successful p38 phosphorylation by LMP1-dependent mechanisms. It must be noted, however, that the overall levels of total p38 were slightly lower in TRADD$^{-/-}$ MEFs, when directly compared to wildtype MEFs. This might contribute to the lower induction of p38 phosphorylation in these cells. Nonetheless, activation of other MAPK pathways like JNK or ERK by NGFR-LMP1wt was also markedly reduced in TRADD$^{-/-}$ MEFs, which argues for a specific partial defect of p38/MAPK pathway activation in the

absence of TRADD. Given that TRAF6 is recruited to LMP1 via the CTAR2 domain, it is consequent to see no p38 phosphorylation, when the CTAR2-mutated receptors NGFR-LMP1($Y_{384}G$) and NGFR-LMP1($A_{204}xAxA/Y_{384}G$) are stimulated by crosslinking (figure 4-12B and C).

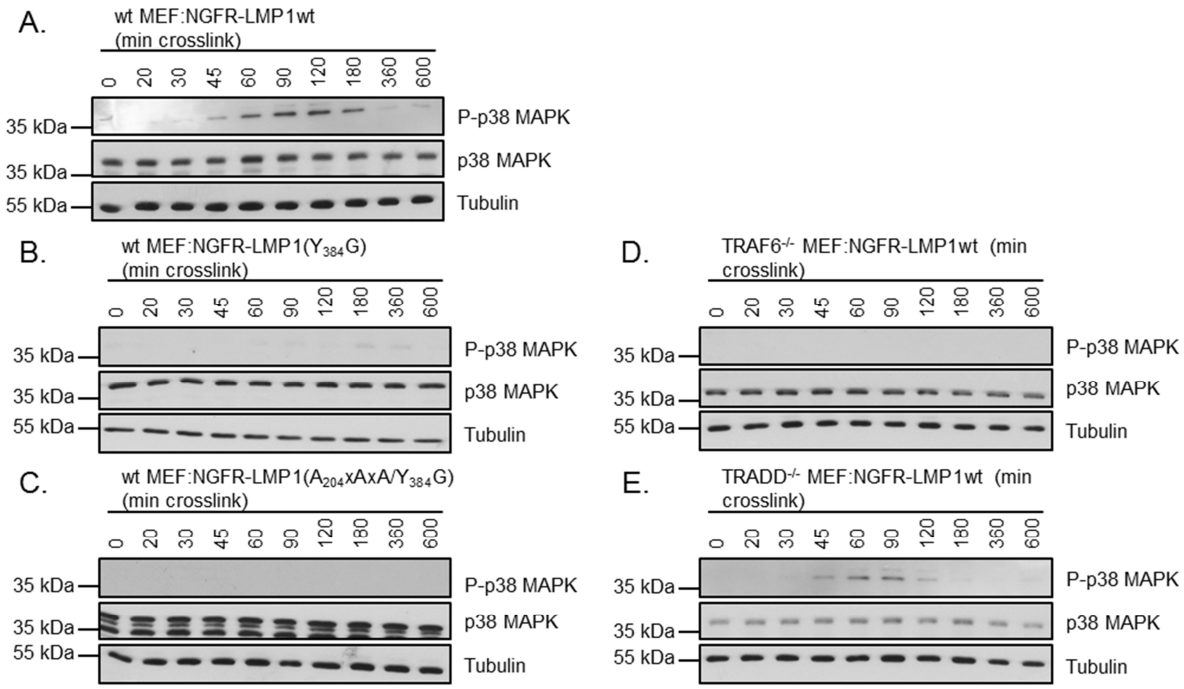

Figure 4-12. Analysis of the p38/MAPK pathway induced by NGFR-LMP1 receptors. Wildtype and knockout MEFs stably expressing NGFR-LMP1wt and mutant receptors as indicated were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml α-fc IgG/IgM at 37°C for the indicated time points between 20 min and 10 h. Induction of the p38/MAPK pathway was analyzed by immunoblotting for phosphorylation of p38 at Thr180 and Tyr182. Tubulin served as a loading control.

In conclusion it was possible to confirm the essential role of TRAF6 for p38/MAPK with the NGFR-LMP1 system in MEFs. Furthermore, a supportive role could be ascribed to TRADD. Clearly, the CTAR2 domain alone was essentially responsible for triggering p38 phosphorylation upon NGFR-LMP1-crosslinking in MEFs.

4.2.4 Activation of ERK1 and ERK2 by LMP1

The ERK MAPK pathway is an important inducer of cell cycle progression, and ERK1 and ERK2 are the most widely studied isoforms. Activation of ERK1/2 is achieved by phosphorylation of the

residues Thr202 and Tyr204. Antibodies directed against both phosphorylation sites were used to assess the activation of ERK1/2 by NGFR-LMP1.

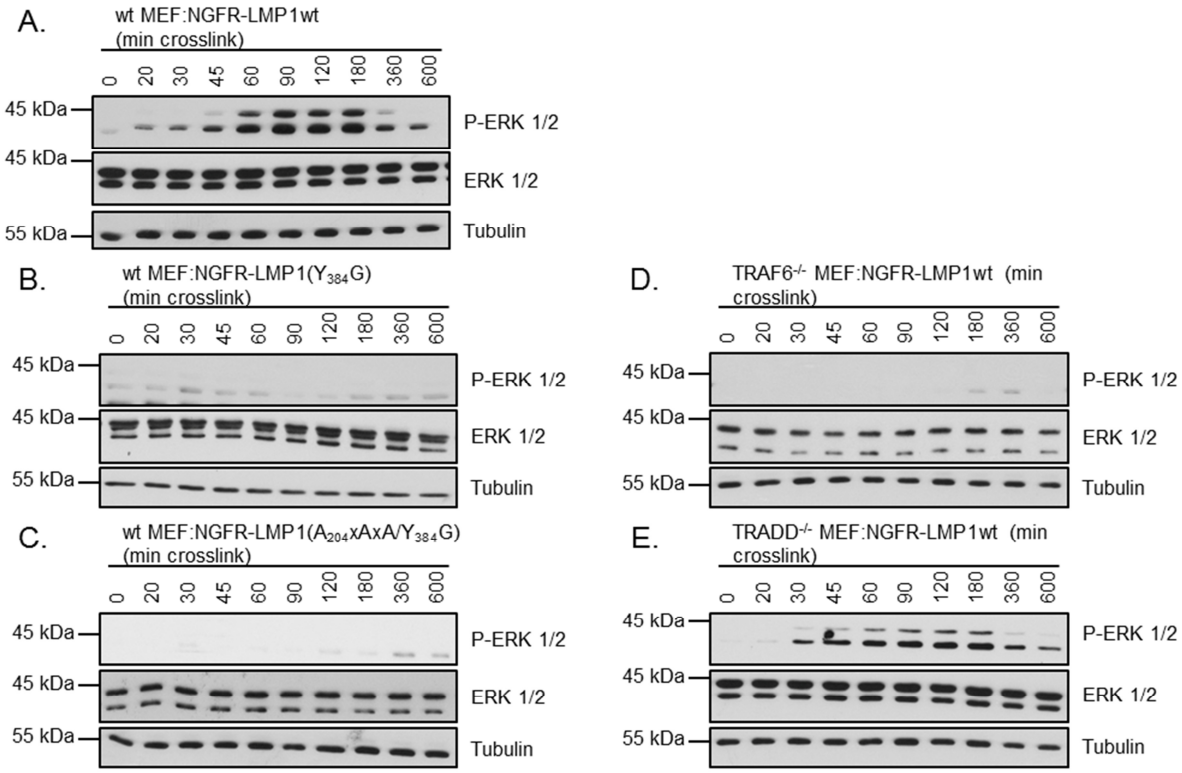

Figure 4-13. Analysis of the ERK pathway induced by NGFR-LMP1 receptors. Wildtype and knockout MEFs stably expressing NGFR-LMP1wt and mutant receptors as indicated were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml α-fc IgG/IgM at 37°C for the indicated time points between 20 min and 10 h. Induction of the ERK pathway was visualized by immunoblotting for phosphorylation of ERK1/2 at Thr202 and Tyr204. Tubulin served as a loading control.

Phosphorylation of the 42 kDa form of ERK (ERK2) occurred as early as 20 minutes after crosslinking stimulation of NGFR-LMP1wt in wildtype MEFs, although a strong band was not detected until 45 to 60 minutes after stimulation. At this time point ERK1 (44 kDa) was also phosphorylated, but never to such a great extent as ERK2 (figure 4-13A). Both forms remained strongly phosphorylated until the signal declined after 180 to 360 minutes of crosslinking. A very similar kinetics were observed in TRADD$^{-/-}$ MEFs (figure 4-13E). Here, ERK2 was also phosphorylated stronger than ERK1 and at earlier time points. However, the overall level of phosphorylated ERK was always lower than in wildtype MEFs, although basal ERK protein levels were comparable. This result demonstrates that TRADD is not critically involved in the activation of ERK by LMP1, but that the lack of TRADD reduces the ability of LMP1 to activate

this MAPK pathway. This observation reflects the induction of the p38- and JNK-pathway in TRADD$^{-/-}$ cells, and hints at a shared mechanism of activation of all three MAPK-pathways concerning the role of TRADD.

So far, most reports attributed the ability of LMP1 to induce the ERK pathway to the CTAR1 domain (Mainou et al, 2007). Therefore it was unexpected to see that the lack of TRAF6 as well as the mutation of CTAR2 both completely abrogated the activation of ERK1 and ERK2 (figure 4-13B and D). This means that activation of the ERK pathway in MEFs critically relies on a functional CTAR2 domain and TRAF6 as a mediator of signaling, which holds true for all tested pathways that originate at CTAR2.

In conclusion it appears that ERK1/2 activation follows a similar pattern as activation of the other tested MAPK pathways. Phosphorylation of ERK1 and ERK2 was depending on a functional CTAR2 domain and the presence of TRAF6, whereas TRADD seems to be involved in a non-essential, supporting role.

4.2.5 Activation of the PI3K/Akt Pathway

The central effector kinase of the PI3K/Akt pathway is Akt, which distributes the primary signal to a wide array of cellular target proteins to influence cell growth and survival. For its full activity Akt must be phosphorylated at two different sites (Thr308 and Ser473) (see introduction 1.5.3). To evaluate the capability of LMP1 to activate the PI3K/Akt pathway, immunoblots were analyzed for Akt phosphorylated at Ser473, which reflects Akt activation.

Stimulation of wt MEF:NGFR-LMP1wt cells by antibody crosslinking resulted in the activation of the PI3K/Akt pathway (figure 4-14A). Phosphorylated Akt was seen as early as 30 to 45 minutes after the initial stimulus and the levels gradually increased after that. However, a significant peak of Akt phosphorylation occurred at a late time point around 180 minutes of crosslinking. After that the levels gradually decreased again. This result might suggest that Akt is activated by LMP1 early upon stimulation, but that a second boost occurs at a later time point.

So far, activation of Akt has mostly been correlated with CTAR1 activity (Dawson et al, 2003; Mainou et al, 2005). Therefore it was unanticipated to see that mutation of the CTAR2 domain (wt MEF:NGFR-LMP1($Y_{384}G$), figure 4-14B) abolished phosphorylation of Akt. The very late, slight increase in phospho-Akt levels at 360 and 600 minutes of crosslinking is not a result of CTAR1-dependent signaling, as the same bands appeared upon crosslinking of the signaling-dead double mutant receptor NGFR-LMP1(A_{204}xAxA/$Y_{384}G$) (figure 4-14C). Even more so, the lack of TRAF6, which is a critical component of any signaling pathway originating at CTAR2, also abrogated activation of Akt by LMP1 (figure 4-14D). The fact that both mutation of CTAR2 and lack of TRAF6 abrogate Akt activation suggests that the successful activation of the

PI3K/Akt pathway critically relies on the presence of the signaling complex at CTAR2 in MEFs, even if CTAR1 is able to directly initiate the pathway in other cell systems, as described in the literature (Dawson et al, 2003; Mainou et al, 2005).

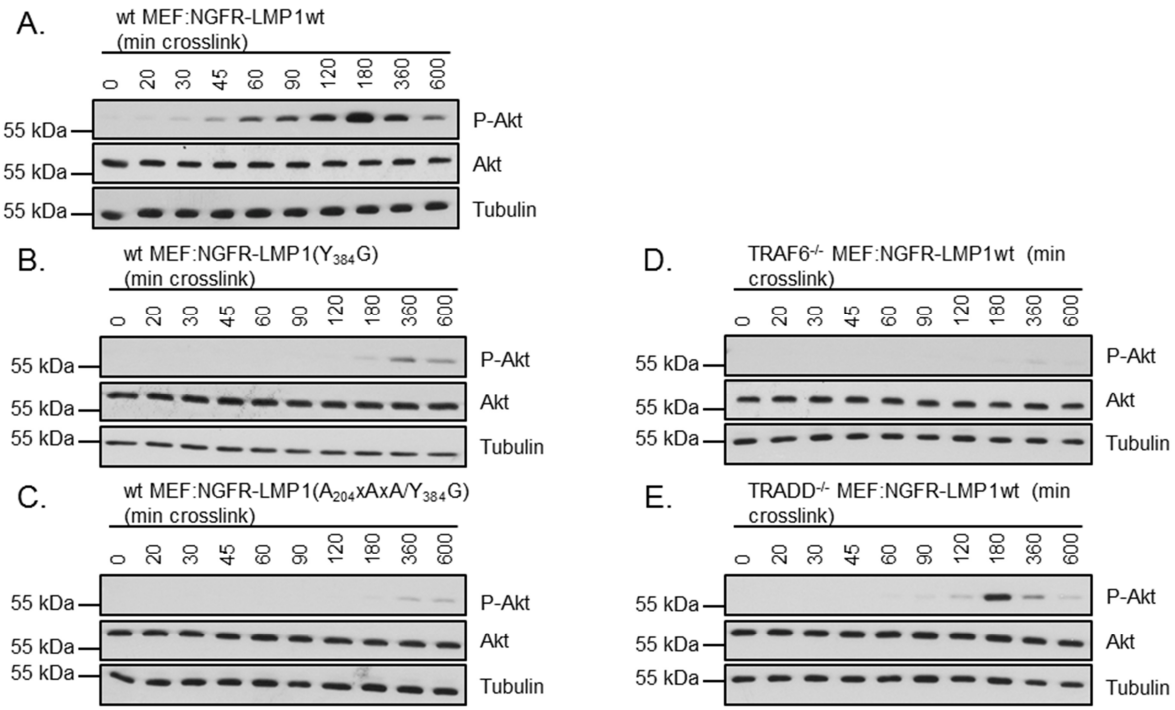

Figure 4-14. Analysis of the PI3K/Akt pathway induced by NGFR-LMP1 receptors. Wildtype and knockout MEFs stably expressing NGFR-LMP1wt and mutant receptors as indicated were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml α-fc IgG/IgM at 37°C for the indicated time points between 20 min and 10 h. Induction of the PI3K/Akt pathway was analyzed by immunoblotting for phosphorylation of Akt at Ser473. Tubulin served as a loading control.

The lack of TRADD did not fully abolish Akt activation upon NGFR-LMP1wt stimulation (figure 4-14E). Here, early phosphorylation of Akt was only weakly detectable, but a strong band appeared after 180 minutes of crosslinking, although it failed to reach the levels observed in wildtype cells. This coincides with the late activation peak of Akt in wt MEF:NGFR-LMP1wt cells, and it shows that TRADD is not critically involved in the activation of Akt by LMP1 in MEFs, but seems to play a non-essential, supportive role.

Taken together, it is obvious that the activation of PI3K/Akt in MEFs upon NGFR-LMP1 stimulation critically relies on CTAR2 and TRAF6, with a minor contribution of TRADD.

4.2.6 Activation of STAT3 by LMP1

Deregulated activity of STAT plays a major role in many tumors and especially in EBV-related diseases, and the mechanisms by which LMP1 triggers STAT activation are only insufficiently understood. Of the STAT-family of proteins, STAT3 plays a major role in NPC, which can be correlated to LMP1 expression (Chen et al, 2003; Lui et al, 2009a; Lui et al, 2009b). Therefore, I investigated the mechanisms of STAT3 activation by inducible NGFR-LMP1 receptors and detected Tyr705-phosphorylated STAT3 in immunoblot experiments.

Phosphorylated STAT3 appeared 60 minutes after crosslinking of NGFR-LMP1wt and peaked between 90 and 120 minutes (figure 4-15A). Thereafter the signal decreased slightly but stayed at a steady level until 600 minutes and the end of the analyzed timeframe. The fact that STAT3 is only activated at relatively late time points suggests that this is not a direct effect of LMP1 signaling, but might rather account for an indirect activation mechanism.

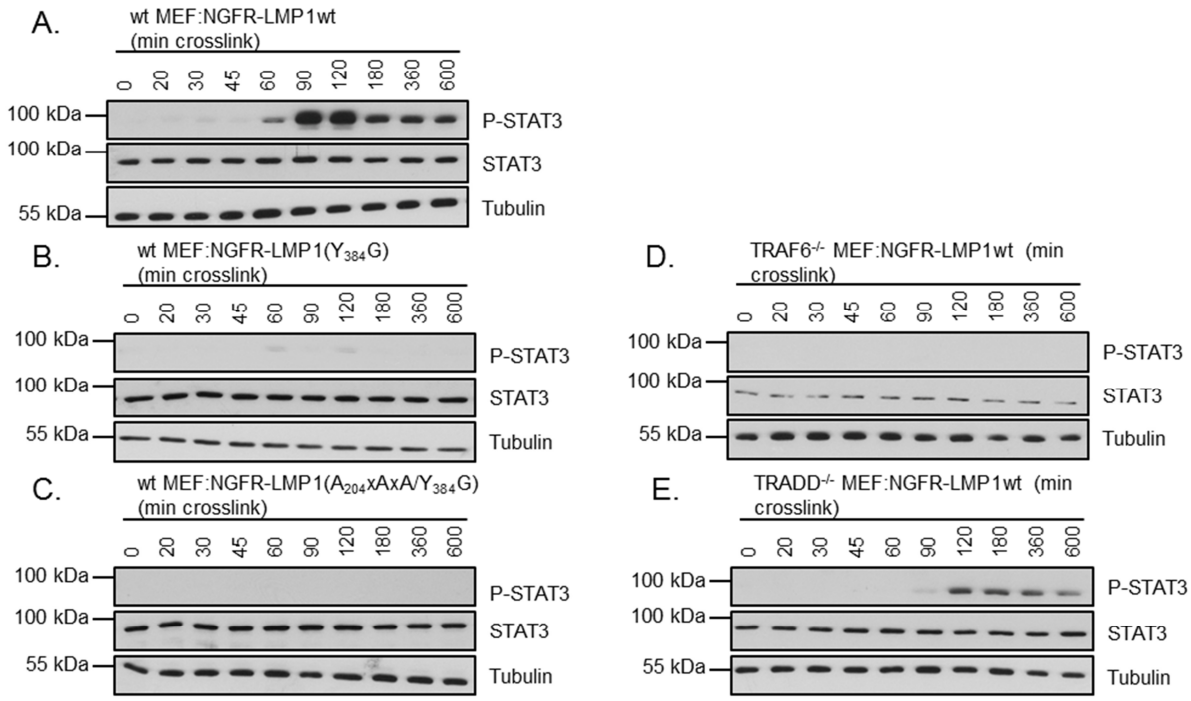

Figure 4-15. Analysis of the JAK/STAT3 pathway induced by NGFR-LMP1 receptors. Wildtype and knockout MEFs stably expressing NGFR-LMP1wt and mutant receptors as indicated were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml α-fc IgG/IgM at 37°C for the indicated time points between 20 min and 10 h. Induction of the Jak/Stat3 pathway was analyzed by immunoblotting for phosphorylation of STAT3 at Tyr705. Tubulin served as a loading control.

No phosphorylated STAT3 was found in wt MEF:NGFR-LMP1($Y_{384}G$) cells upon stimulation (figure 4-15B). The same was true for cells lacking TRAF6, although total STAT3 levels are also lower in $TRAF6^{-/-}$ MEFs compared to wildtype MEFs (figure 4-15D). These findings show for the first time that the activation of STAT3 by LMP1 critically depends on the CTAR2 domain and TRAF6 as a mediator.

Lack of TRADD weakened the ability of LMP1 to activate STAT3, but did not completely abrogate it, since phosphorylated STAT3 was observed in $TRADD^{-/-}$ MEF:NGFR-LMP1wt cells between 90 and 600 minutes of crosslinking (figure 4-15E). This indicates that the presence of TRADD contributes to the activation of STAT3 by LMP1, but it is not a critical mediator.

Taken together, the data suggest that the LMP1-dependent activation of STAT3 critically depends on CTAR2-mediated mechanisms. TRAF6 is a key player in LMP1-induced STAT3 phosphorylation, and TRADD contributes to this effect, but is not a critical factor. Furthermore, STAT3 activation occurs only at later time points of NGFR-LMP1 stimulation, suggesting that this is not a direct effect of LMP1 signaling.

4.2.7 Induction of the Non-Canonical NF-κB Pathway by LMP1

As described in the introduction (chapter 1.5.1), a hallmark of non-canonical NF-κB signaling is the liberation of p52 by cleavage of p100. To investigate the activation of the non-canonical NF-κB pathway by NGFR-LMP1, immunoblots were probed with antibodies detecting both p100 and the cleavage product p52. Induction of the signal should result in a decrease of total p100 and an increase of total p52. Activity of the pathway is indicated by the appearance of p52.

As can be seen in figure 4-16A, p52 was present in wt MEF:NGFR-LMP1wt even in the absence of a stimulus. At the same time, p100 levels were low compared to p52 levels, suggesting that the degradation of p100 to p52 is initialized in these cells already without crosslinking of NGFR-LMP1. The same effect was observed in wt MEF:NGFR-LMP1($Y_{384}G$), $TRADD^{-/-}$ MEF:NGFR-LMP1wt and $TRAF6^{-/-}$ MEF:NGFR-LMP1wt cells (figure 4-16B, D and E). In all three cell lines constitutively high levels of p52 were present at all time points of the stimulation, as well as in the absence of antibody stimulation. The effect was less pronounced in the cell lines lacking TRADD or TRAF6 compared with the wildtype MEFs. However, also here a constitutive p100 cleavage was observed. At the same time, crosslinking had no significant effect on the degradation of p100 in wildtype or knockout MEFs, suggesting that stimulation of NGFR-LMP1 could not further increase the activation of the non-canonical NF-κB pathway. The constitutive degradation of p100 to p52 was dependent on the presence of the TRAF binding motif within the CTAR1 domain of LMP1. This assumption is strongly supported by the results obtained with wt MEF:NGFR-LMP1(A_{204}xAxA/$Y_{384}G$) (figure 4-16C). Here, the levels of p100 were high and no

p52 was found at any time point during the stimulation. This demonstrates that no constitutive or inducible degradation of p100 to p52 takes place when the TRAF-binding motif in CTAR1 is mutated.

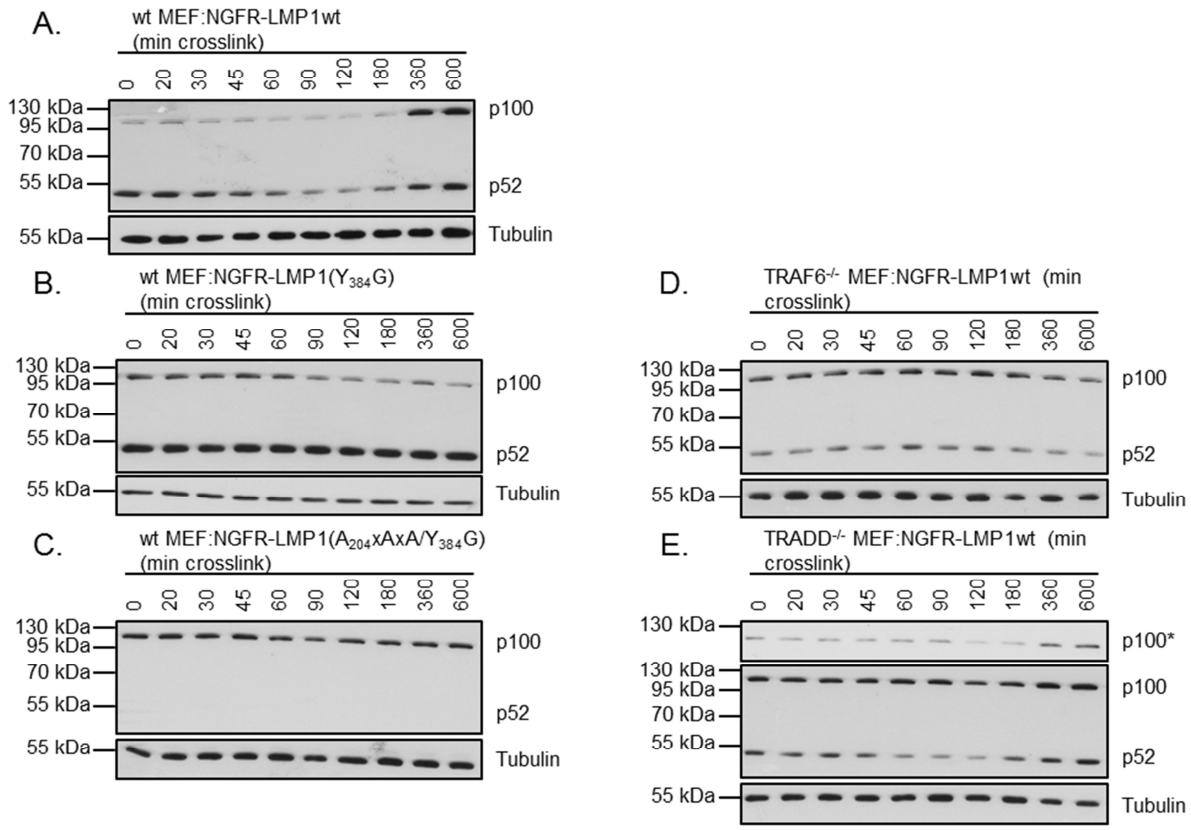

Figure 4-16. Analysis of the non-canonical NF-κB pathway induced by NGFR-LMP1 receptors. Wildtype and knockout MEFs stably expressing NGFR-LMP1wt and mutant receptors as indicated were stimulated by NGFR-crosslinking as described before. Induction of the non-canonical NF-κB pathway was analyzed by immunoblotting for the degradation of p100 to p52. Tubulin served as a loading control. (*) indicates a shorter exposure.

Considering that no constitutive activation of other signaling pathways like the canonical NF-κB, JNK or PI3K/Akt pathway was detected in the absence of a stimulus, as described in the previous chapters, the CTAR1-dependent constitutive activation of the non-canonical NF-κB pathway was unexpected. It is possible that the activation threshold for this pathway by LMP1 is much lower than for other pathways or that LMP1 induces some sort of “tonic” signaling to non-canonical NF-κB that does not rely on forced oligomerization of the receptor.

The gene encoding for the p100 precursor protein itself is a target of the canonical NF-κB pathway, which is also true for LMP1-induced signaling (Atkinson et al, 2003). As anticipated,

stimulation of NGFR-LMP1wt in wildtype MEFs led to increased levels of p100 after 360 and 600 minutes of stimulation (figure 4-16A). At the same time, p52 levels also slightly increased, which is likely a consequence of the rising levels of the p100 precursor. Consistent with the mandatory role for CTAR2 and TRAF6 in canonical NF-κB signaling, no increase in p100 levels was observed in cells lacking TRAF6 or expressing NGFR-LMP1 with the $Y_{384}G$ mutation (figure 4-16B, C and D). Lack of TRADD diminished the increase in p100, but did not completely abrogate it (figure 4-16E). This is again consistent with a contributing role for TRADD in CTAR2-dependent signaling.

4.2.8 LMP1-Induced Effects on TRAF3

TRAF3 is an important inhibitor of the non-canonical NF-κB pathway, as described in the introduction (chapter 1.5.1), and the degradation of TRAF3 is usually a hallmark of the activation of the non-canonical NF-κB pathway (Vallabhapurapu et al, 2008). TRAF3 has been described to be recruited to LMP1 via the P_{204}xQxT TRAF-binding motif in CTAR1 (Devergne et al, 1996; Xie & Bishop, 2004), and overexpression of TRAF3 could prevent LMP1-induced processing of p100 (Song & Kang, 2010), indicating a role for TRAF3 in LMP1-mediated activation of the non-canonical NF-κB pathway. Since the constitutive processing of p100 to p52 was dependent on the CTAR1 domain upon NGFR-LMP1 crosslinking, the question arose whether TRAF3 was affected by signaling events originating at CTAR1, and if this was also a constitutive event. Therefore the ability of LMP1 to affect TRAF3 in the presence and absence of NGFR-LMP1 stimulation was tested. For this, a more in-depth experiment was conducted and all NGFR-LMP1wt and mutant receptors were examined in combination with TRAF6 and TRADD knockout cell lines upon stimulation for 60 and 120 minutes. Cleared NP-40 whole cell lysates were separated by SDS-PAGE and TRAF3 protein levels were examined by immunoblotting.

Upon stimulation of NGFR-LMP1, TRAF3 levels rapidly decreased (figure 4-17A – C). This could be seen as long as the P_{204}xQxT motif in CTAR1 was intact, and TRAF3 levels dropped at 60 and 120 minutes after crosslinking of either NGFR-LMP1wt or NGFR-LMP1($Y_{384}G$). This was true for wildtype cells as well as MEFs lacking TRAF6 or TRADD, suggesting that none of these proteins needs to be present to induce LMP1-dependent TRAF3 decrease (figure 4-17A – C, far left and middle right). No TRAF3 decrease was observed upon crosslinking of NGFR-LMP1(A_{204}xAxA) or NGFR-LMP1(A_{204}xAxA/$Y_{384}G$) irrespective of the cellular background, although NGFR-LMP1 protein levels were also lower in those cases (figure 4-17A – C, far right and middle left). Nonetheless, it is clear that TRAF3 reduction must rely on CTAR1, because NGFR-LMP1($Y_{384}G$), which only features a functional CTAR1 domain, while the CTAR2 domain is mutated, potently induced TRAF3 depletion. The same was true for NGFR-LMP1 signaling in

TRAF6$^{-/-}$ MEFs or TRADD$^{-/-}$ MEFs, in which the lack of TRAF6 and TRADD blocked or reduced CTAR2 dependent signaling, respectively.

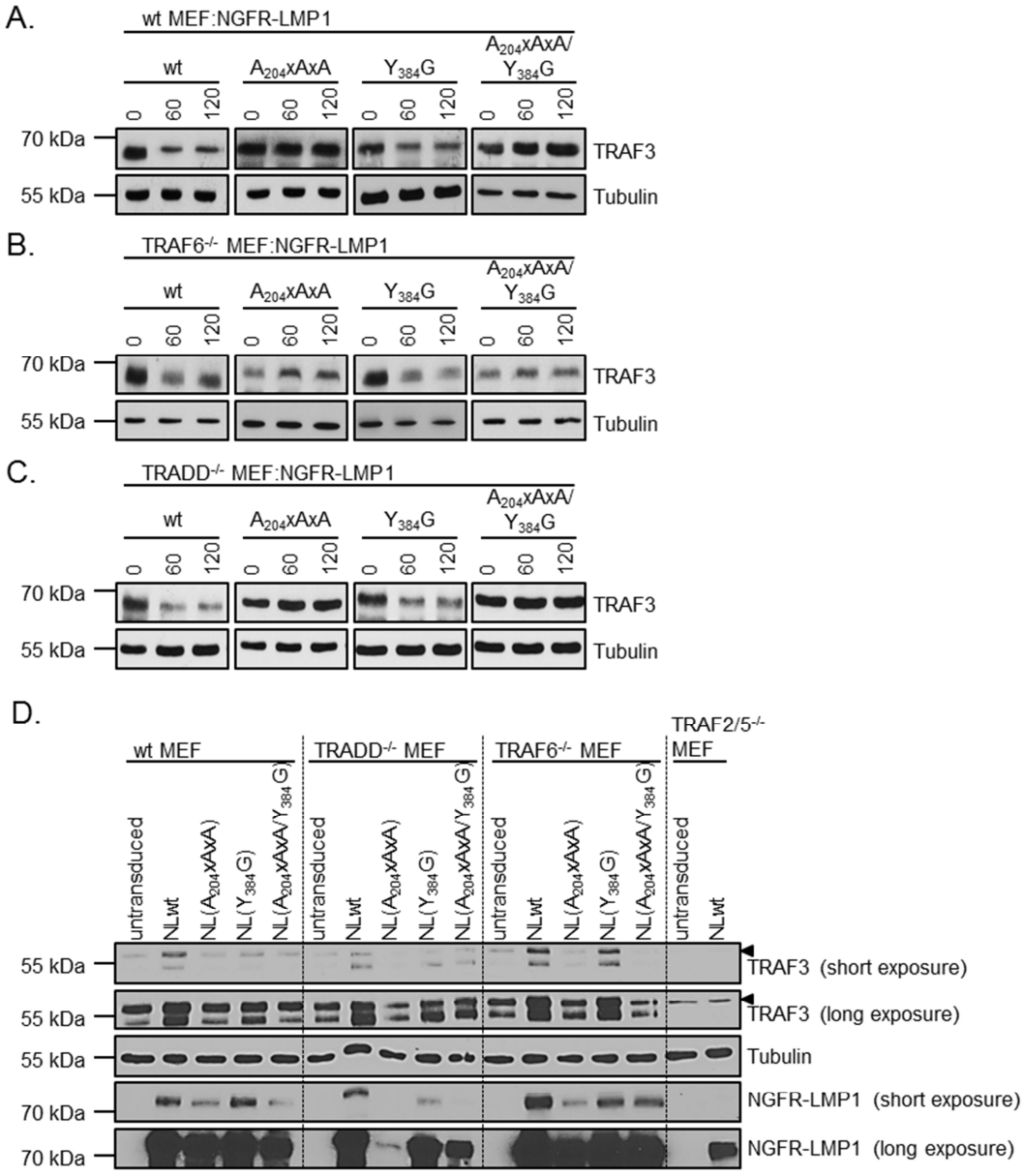

Figure 4-17. Reduction of TRAF3 protein levels by NGFR-LMP1 critically relies on the CTAR1 domain. A-C. Wildtype and knockout MEFs stably expressing NGFR-LMP1wt and mutant receptors as indicated were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C followed by crosslinking with 10 µg/ml α-fc IgG/IgM at 37°C for the indicated time points of 60 and 120 min, before NP-40 lysates were prepared and cleared by centrifugation. Immunoblotting for TRAF3 was used to assay the induction of the non-canonical NF-κB pathway, which is hallmarked by the degradation of TRAF3. Tubulin served as a loading control. **D.** Basal TRAF3 expression in all indicated cell lines was assessed by immunoblotting for total TRAF3. Tubulin served as a loading control and NGFR-LMP1 levels were visualized with the help of anti-LMP1 antibodies (3G6-1).

Notably, basal TRAF3 levels differed greatly among the cell lines as well. Unstimulated wildype MEFs as well as MEFs deficient in TRADD or TRAF6 expressing NGFR-LMP1wt receptors yielded higher TRAF3 levels than the corresponding MEF cell lines that were not transduced (figure 4-17D). This means that the basal, constitutive activity of the receptor is sufficient to deregulate TRAF3 protein levels in these cells. The effect was most pronounced in TRAF6-deficient MEFs, but here NGFR-LMP1wt levels were also slightly higher compared to wildtype and TRADD$^{-/-}$ MEFs, which may account for the stronger effects on TRAF3.

It is likely that this effect on basal TRAF3 expression orginates at CTAR1. Deficiency of TRAF6, which is a critical mediator of signaling at CTAR2, did not abolish elevation of TRAF3 levels (figure 4-17D). Additionally, expression of NGFR-LMP1 with a mutation in CTAR2 ($Y_{384}G$) also led to elevated TRAF3 levels, which was most prominent in TRAF6$^{-/-}$ MEFs (figure 4-17D).

In conclusion, the immunoblot results for p100 and TRAF3 suggest four major points: (1) NGFR-LMP1 induces constitutive processing of p100 to p52, even in the absence of a stimulus. This means that no forced oligomerization of the receptor is needed for signal induction, but that possibly the abundance of the receptor on the cell surface is sufficient to overcome the threshold for pathway activation, or NGFR-LMP1 is active as a monomer. (2) Since no other pathway was constitutively induced by the sheer presence of NGFR-LMP1 on the cell surface, the threshold for induction of the non-canonical NF-κB pathway must be considered to be very low compared to e.g. the canonical NF-κB pathway. (3) Constitutive activation of the non-canonical NF-κB pathway by NGFR-LMP1 is critically dependent on the TRAF-binding site in CTAR1. (4) This constitutive activation of the non-canonical NF-κB pathway by NGFR-LMP1 might not depend on TRAF3. LMP1-crosslinking inducibly affected TRAF3 in a way that the protein levels were reduced in the immunoblot after crosslinking in a CTAR1-dependent manner. However, no further processing of p100 was achieved by this. Furthermore, TRAF3 levels were elevated in cells expressing NGFR-LMP1wt, likely as a result of constitutive signaling, but these elevated TRAF3 levels had no negative effect on the constitutive processing of p100.

4.2.9 TRAF3 Is Not Degraded in the Context of LMP1 Signaling, but Translocated

It has been proposed that LMP1, in contrast to CD40, does not induce the degradation of TRAF3 in murine B cells (Brown et al, 2001). This would mean that the decrease of TRAF3 upon NGFR-crosslinking in the blots shown in figure 4-17A – C is not due to the degradation of the protein. Instead, it is possible that TRAF3 is rather translocated to an NP-40-insoluble fraction upon LMP1 stimulation. To test this hypothesis, crosslinking experiments were conducted in the presence and absence of the proteasome inhibitor MG132, and both the soluble lysate fraction and insoluble pellet fraction of the cells were examined for TRAF3 in immunoblot experiments.

Cells expressing NGFR-LMP1wt were used in the experiment. Additionally, the impact of the anti-NGFR antibody-incubation alone was investigated to rule out that anti-NGFR binding alone is sufficient to induce activation of the receptor and TRAF3 depletion.

TRAF3 levels decreased after 1 h crosslinking of NGFR-LMP1wt (figure 4-18A). In line with the data published by Brown et al. (Brown et al, 2001), this decrease occurred both in the absence and presence of MG132, indicating that the observed reduction in protein levels was not due to degradation by the proteasome. Instead, TRAF3 was found in the NP-40-insoluble pellet of the cell lysate, and these levels increased greatly after crosslinking-stimulation. This strongly suggests that LMP1 signaling induces a translocation of TRAF3 into the sub-cellular fraction residing in the insoluble pellet after cell lysis rather than its degradation.

Notably, the reduction of TRAF3 in the soluble fraction was not induced by the binding of anti-NGFR antibody to NGFR-LMP1 alone, but depended on induced crosslinking by the secondary antibody (figure 4-18A). TRAF3 protein levels in the soluble fractions were similar comparing unstimulated cells and those incubated with anti-NGFR alone, confirming that no effect on TRAF3 was achieved by incubation of the cells with anti-NGFR alone (figure 4-18A).

It could be argued that the shift of TRAF3 into the insoluble fraction reflects not the inducible association of TRAF3 with LMP1 upon crosslinking, but rather a simultaneous translocation of a preformed complex of NGFR-LMP1 and TRAF3 into insoluble compartments after crosslinking, and that this preformed complex is responsible for the constitutive activation of the non-canonical NF-κB pathway. However, NGFR-LMP1 was present in both the soluble insoluble pellet fraction regardless of crosslinking, and no simultaneous increase of NGFR-LMP1 and TRAF3 in the pellet fraction was observed. Furthermore, it was shown that LMP1 translocates into the lipid raft fraction upon activation and associates with TRAF3 inside these rafts, but no recruitment of TRAF3 takes place outside of lipid rafts (Ardila-Osorio et al, 1999; Ardila-Osorio et al, 2005; Briseno-Franke, 2006; Brown et al, 2001). To test if NGFR-LMP1 is present in lipid rafts prior to crosslinking, wt MEF:NGFR-LMP1wt cells were stimulated by crosslinking or left untreated, and were prepared afterwards to isolate the lipid rafts fraction. No NGFR-LMP1 was found in lipid rafts (fraction 2, R) in unstimulated cells, but a translocation into rafts was observed in the cells subjected to 1 h crosslinking (figure 4-18B). This proves that NGFR-LMP1 is not associated with lipid rafts in the absence of antibody-crosslinking, but that crosslinking induces a shift into these compartments.

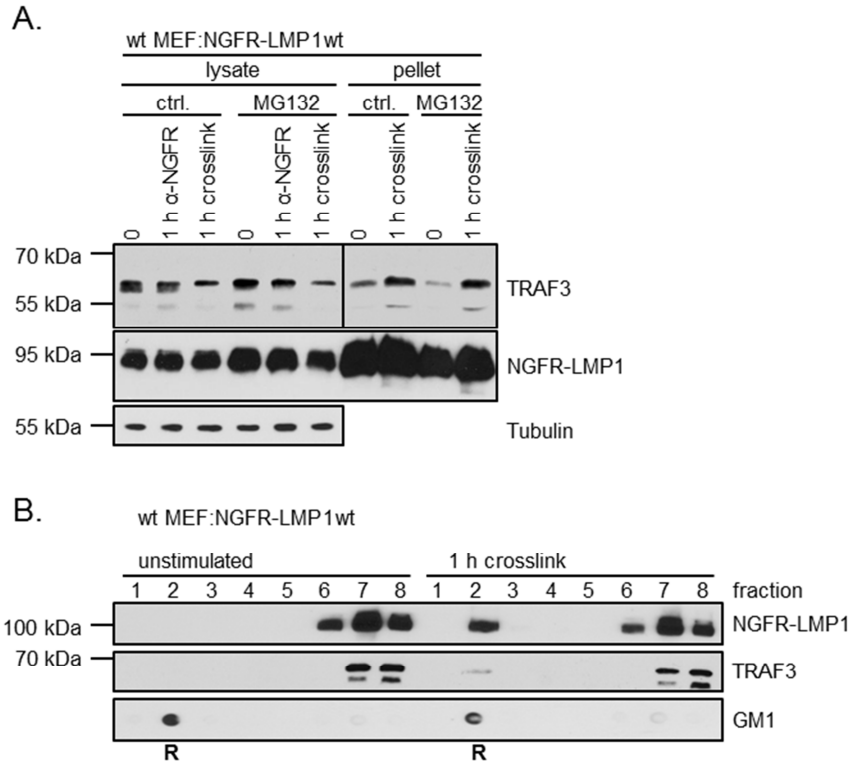

Figure 4-18. TRAF3 is not degraded, but translocates to the NP-40-insoluble fraction upon NGFR-LMP1 crosslinking, and translocates with LMP1 to lipid rafts. A. Wildtype MEFs stably expressing NGFR-LMP1wt were incubated with anti-NGFR (1 µg/ml) for 1 hour (1 h α-NGFR), or left untreated (0). To induce crosslinking the incubation with α-NGFR was followed by 1 hour crosslinking with 10 µg/ml IgG/IgM (1 h crosslink) in the presence or absence of 20 µM MG132. The cells were lysed with 0.1 % NP-40 lysis buffer and both the cleared lysate and the insoluble pellet were separated by SDS-PAGE. Immunoblots were stained for TRAF3, LMP1 and Tubulin as a loading control. **B.** wt MEF:NGFR-LMP1wt were incubated with anti-NGFR (1 µg/ml) for 1 hour followed by 1 hour crosslinking with 10 µg/ml IgG/IgM or left untreated, before fractions were prepared to isolate lipid rafts. Fraction 2 containing the lipid rafts (indicated by **R**) was visualized on a dot blot stained for GM1 with cholera toxin subunit B. Fractions were separated by SDS-PAGE and NGFR-LMP1 and TRAF3 were visualized by immunoblot.

At the same time, TRAF3 was found in the lipid raft fraction together with activated NGFR-LMP1 (figure 4-18B), which confirms previously published data and shows that an association of NGFR-LMP1 and TRAF3 in lipid rafts is induced upon NGFR-crosslinking. Since TRAF3 was shown to associate with LMP1 only inside lipid rafts (Briseno-Franke, 2006), it must be considered that no preformed complex of the two proteins exists prior to crosslinking.

Taken together, these data strongly indicate that LMP1 is capable of inducing the non-canonical NF-κB pathway by a mechanism that differs from other receptors, concerning the fate of TRAF3. Upon activation of the receptor TRAF3 translocates to NP-40 insoluble cell compartments rather

than degraded by the proteasome. At the same time the two observations that (1) NGFR-LMP1 activates p100 degradation in the absence of stimulation and that (2) TRAF3 is depleted from the cytosol and translocates into the insoluble fraction and into lipid rafts only upon NGFR-crosslinking stimulation, together suggest that LMP1 is capable of inducing the non-canonical NF-κB pathway in a CTAR1-dependent manner that is independent of TRAF3. Instead, other mechanisms might be responsible for the activation of IKK1 or p100.

4.3 Reconstitution of TRAF6

TRAF6 is indispensable for many signaling functions of LMP1 and plays a critical role in CTAR2-dependent signaling. The JNK pathway, the p38/MAPK and the canonical NF-κB pathway all critically rely on TRAF6 (figures 4-10D, 4-11D and 4-12D), but the protein also seems to be important for the LMP1-dependent activation of Akt, ERK and STAT3 (figures 4-13D, 4-14D and 4-15D). Therefore, the question arose if these defects were due to the absence of TRAF6, or if other TRAF6-unrelated defects in the knockout cells led to the observed effects. To test this TRAF6 expression was rescued in the knockout cells.

I aimed at rescuing TRAF6$^{-/-}$ MEF:NGFR-LMP1wt cells expressing low levels of NGFR-LMP1 (GFP low, see chapters 4.1.3 and 4.1.4) with Flag-tagged wildtype TRAF6, to prove that expression of TRAF6 would rescue the induction of CTAR2 dependent signaling pathways by NGFR-LMP1wt. To establish TRAF6$^{-/-}$ MEF:NGFR-LMP1wt/Flag-TRAF6wt cells, Flag-TRAF6 was introduced into TRAF6$^{-/-}$ MEF:NGFR-LMP1wt cells by retroviral transduction. Similar to the retroviral vector encoding for NGFR-LMP1 (see chapter 4.1.3), the sequence encoding for Flag-TRAF6wt was cloned into the SF91-IRES-GFP-WPRE retroviral vector (Schwieger et al, 2002) (figure 4-19A). Because NGFR-LMP1-expressing cells already carried a GFP gene due to the first transduction event (see chapter 4.1.3), the GFP-sequence in the rescue-vector was replaced by the sequence encoding for cyan fluorescent protein (CFP). Thereby the rescued cells stably expressing the LTR-element containing the TRAF6-sequence could later be enriched by sorting for CFP.

Reintroducing a protein into a cell can lead to problems evoked by the overexpression of that protein (Prelich, 2012). The transduced TRAF6 is not transcribed from the endogenous promoter, but from the retroviral LTR. This promoter is relatively strong compared to the TRAF6 promoter. To achieve the lowest possible TRAF6 expression, the transduced cells were sorted for low CFP expression, similar to the enrichment of cells expressing low GFP levels in the context of NGFR-LMP1 expression (see 4.1.3). However, even cells sorted for low CFP still highly overexpressed TRAF6 compared to the endogenous levels in wildtype MEFs (figure 4-19B), and it was not possible to further reduce Flag-TRAF6 levels.

It is known that overexpression of TRAF6 can lead to constitutive background activation of certain signaling pathways such as the NF-κB pathway (Baud et al, 1999; Luftig et al, 2003). To control whether the stable expression of TRAF6 after retroviral transduction would lead to such side-effects, TRAF6$^{-/-}$ MEFs that did not express NGFR-LMP1 were rescued in the same manner with Flag-TRAF6wt. However, no unspecific activation of the canonical NF-κB pathway or the JNK pathway was observed in these cells, and similarly, no significant background signaling was induced in unstimulated TRAF6-rescued TRAF6$^{-/-}$ MEF:NGFR-LMP1wt cells (data not shown and figure 4-19B).

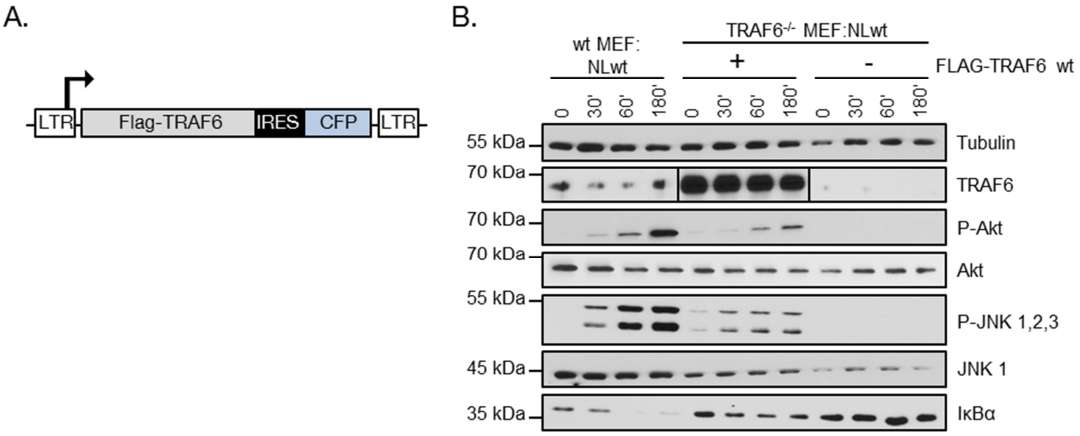

Figure 4-19. The canonical NF-κB pathway, the JNK pathway and the induction of the PI3K/Akt pathway by NGFR-LMP1 can be rescued by Flag-TRAF6. TRAF6$^{-/-}$ MEFs stably expressing the NGFR-LMP1wt receptor were superinfected with a retrovirus for the stable expression of Flag-tagged TRAF6wt. **A.** Schematic overview of the retroviral construct used for the TRAF6 reconstitution. The 5' LTR cassette served as a promoter for the Flag-TRAF6wt gene, which is directly followed by an IRES site, giving access to the subsequent CFP gene. Cells were therefore FACS-sorted for CFP expression, which correlates with Flag-TRAF6 expression. **B.** Wildtype MEF and TRAF6$^{-/-}$ MEF with or without reconstituted Flag-TRAF6 expressing NGFR-LMP1wt were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml α-fc IgG/IgM at 37°C for the indicated time points of 30, 60 and 180 min. Antibodies against Akt phosphorylated at Ser473, JNK phosphorylated at Thr182 and Tyr 184, as well as IκBα were used to assess the induction of the PI3K/Akt pathway, the JNK pathway and the canonical NF-κB pathway. Tubulin served as a loading control.

To test the ability of TRAF6$^{-/-}$ MEF:NGFR-LMP1wt/Flag-TRAF6wt cells to respond to induction of LMP1 signaling, crosslinking experiments were conducted. As a positive and negative control wt MEF:NGFR-LMP1wt cells and TRAF6$^{-/-}$ MEF:NGFR-LMP1wt cells were used, respectively. As shown in figure 4-19B, TRAF6 was highly expressed in the cells rescued with Flag-TRAF6 compared to the endogenous levels in wildtype MEFs. Antibody crosslinking of NGFR-LMP1 led

to the activation of Akt and JNK and the degradation of IκBα in the TRAF6-reconstituted cells with similar kinetics compared to the wt MEF:NGFR-LMP1wt control. However, the overall activation of the three pathways seemed to be not as strong as in the wildtype MEFs (figure 4-19B). This effect might be due to the different expression levels of TRAF6 in wildtype and rescued cells. Overexpression of a protein naturally disrupts the stoichiometry of signaling complexes containing that protein, which might lead to deregulated signaling. It is possible that the high amounts of TRAF6 ultimately reduce the signal outcome due to stoichiometric issues, a phenotype that was described for example for the overexpression of histones (Prelich, 2012). This could also be the reason for the lack of STAT3 or ERK phosphorylation after stimulation of the TRAF6-reconstituted cells (data not shown). However, STAT3 activation and partially ERK and Akt activation by NGFR-LMP1 critically relied on indirect signaling mediated by JNK, canonical NF-κB and p38/MAPK, as shown later in chapter 4.4. It is possible that induction of JNK and NF-B by NGFR-LMP1 in the TRAF6-rescued MEFs is not sufficient to induce indirect STAT3 or ERK activation, while the observed Akt phosphorylation might be a direct signaling effect. Basal protein levels also differed between TRAF6$^{-/-}$ and wildtype MEFs. STAT3 and JNK1 are expressed at lower levels in TRAF6$^{-/-}$ MEFs than in wildtype MEFs (figures 4-15A and D and 4-19B). JNK1 levels increased after reconstitution of TRAF6$^{-/-}$ MEFs with Flag-TRAF6wt, although the levels stayed below the JNK1 expression levels in wildtype MEFs (figure 4-19B).

The presented results demonstrate that despite the high exogenous TRAF6 expression, it is possible to partially rescue the activation of JNK, Akt and canonical NF-κB pathways by reintroducing Flag-TRAF6wt into the TRAF6$^{-/-}$ MEF:NGFR-LMP1wt cells. This confirms that the defects observed after NGFR-LMP1wt crosslinking in TRAF6$^{-/-}$ MEFs were indeed resulting from the lack of TRAF6, and were not due to off-target effects. Furthermore, the successful rescue provides a basis for further rescue attempts using TRAF6 mutants, which can elucidate the role of TRAF6 in LMP1 signaling in more detail

4.4 Indirect Signaling Mechanisms of LMP1

4.4.1 LMP1 Induces the Activation of Akt, ERK and STAT3 through Indirect Mechanisms

Two aspects of the results from the systematical crosslinking experiments were especially striking and interesting. First, the activation of both Akt and ERK was critically depending on CTAR2 and TRAF6 (chapters 4.2.4 and 4.2.5). This was not anticipated since so far both pathways had been primarily described to be initiated at CTAR1 (Dawson et al, 2003; Mainou et al, 2005; Mainou et al, 2007). Second, strong activation of both Akt and STAT3, and to a lesser extent of ERK occurred only at relatively late time points during the crosslinking kinetics (chapters 4.2.4, 4.2.5 and 4.2.6). STAT3 was effectively phosphorylated at around 90 and 120

minutes of crosslinking (figure 4-15A), and strong phosphorylation of Akt was never observed before an even later time point around 180 minutes (figure 4-14A). Similarly, ERK phosphorylation was strongest between 90 and 180 minutes of stimulation (figure 4-13A). These late activation peaks suggest that the underlying mechanisms might not be induced directly by LMP1, but rather by an indirect mechanism.

To test the possibility that LMP1 initiates the activation of STAT3, Akt and ERK through steps involving protein synthesis, crosslinking experiments in the presence or absence of cycloheximide were conducted. Cycloheximide specifically inhibits protein synthesis in eukaryotic cells by interfering with translational elongation of a nascent protein. By using this compound, any de novo protein synthesis, which is induced by LMP1-dependent pathways upon crosslinking, is blocked.

Crosslinking of NGFR-LMP1 resulted in strong phosphorylation of STAT3 after 90 minutes with the same kinetics as described before (chapter 4.2.6). Importantly, no STAT3 phosphorylation was observed upon NGFR-LMP1 crosslinking in the presence of cycloheximide (CHX) (figure 4-20A). A faint band appeared after 180 minutes of crosslinking with cycloheximide, but this band was also present in the control “CHX only” treatment, in which the cells were not stimulated by antibody-crosslinking. Therefore, this effect is not induced by LMP1. The complete lack of phosphorylated STAT3 in the presence of cycloheximide suggests that the activation of STAT3 is an entirely indirect event, which relies critically on the de novo synthesis of secondary factors, in contrast to a direct JAK/STAT3 activation. Similarly, the late, enhanced phosphorylation of Akt was significantly reduced in the presence of cycloheximide (figure 4-20B), meaning that full Akt activation by LMP1 depends on the upregulation of an unknown factor. However, LMP1 was capable of activating Akt to a certain extent in a direct manner that does not rely on de novo protein synthesis and likely reflects direct activation by CTAR1. The same is true for the activation of ERK (figure 4-20C). ERK was phosphorylated through LMP1-dependent mechanisms in the presence of cycloheximide, but no enhanced phosphorylation occurred between 120 and 180 minutes of crosslinking.

The inhibitory effect of cycloheximide treatment was specific for the activation of STAT3, Akt and ERK. Degradation of IκBα or phosphorylation of JNK and p38 were not reduced by cycloheximde (figure 4-20D). This demonstrates that those three pathways are activated in an exclusively direct manner and that they are not affected in a negative way by the inhibition of protein synthesis. Additionally, IκBα levels did not recover after crosslinking-stimulation in the presence of cycloheximide, which demonstrates that protein synthesis was successfully blocked by the compound. JNK and p38 phosphorylation were not negatively affected by cycloheximide treatment, however, cycloheximide seemed to augment the LMP1-induced activation of both

pathways (figure 4-20D). It has been shown before that cycloheximide induced JNK and p38 responses in different experimental systems, which indicates that this might be a natural stress response (Chang et al, 2013; Itani et al, 2003; Seymour et al, 2006).

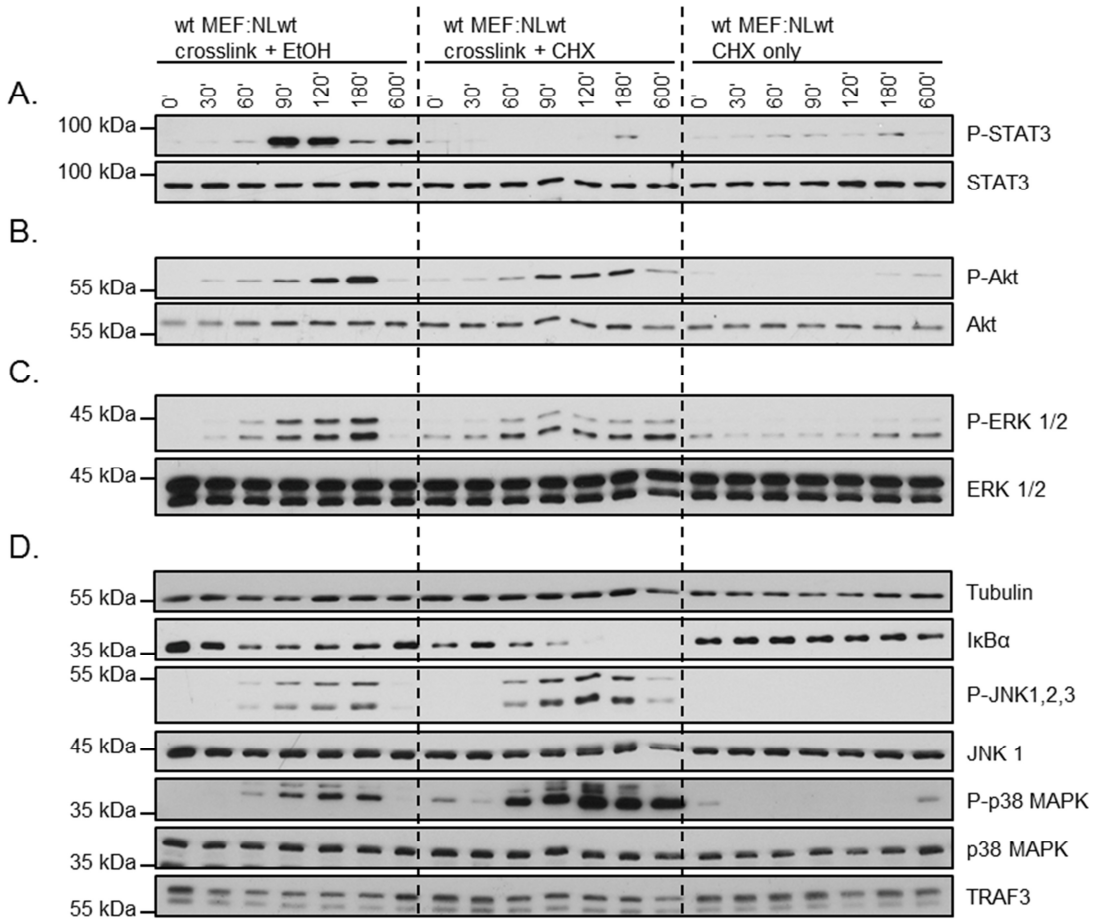

Figure 4-20. Cycloheximide treatment abrogates the phosphorylation of STAT3 and diminishes the induction of the PI3K/Akt and ERK pathways by NGFR-LMP1. Wildtype MEFs stably expressing NGFR-LMP1wt (NLwt) were stimulated by staining with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml α-fc IgG/IgM at 37°C for the indicated time points in the presence or absence of 25 µM cycloheximide (CHX). As a control, cells were also treated with 25 µM CHX only. To analyze the activation of the STAT3, the PI3K/Akt and the ERK pathways, immunoblots for STAT3 phosphorylated at Tyr705 (**A.**), Akt phosphorylated at Ser473 (**B.**) and phosphorylation of ERK1/2 at Thr202 and Tyr204 (**C.**) were performed. Tubulin served as a loading control, and the induction of the JNK pathway, the canonical NF-κB pathway, the p38/MAPK pathway and the non-canonical NF-κB pathway were analyzed to control any major side effects of the cycloheximide treatment on the cells and the crosslinking kinetics (**D.**).

In summary these results show that the activation of STAT3 by LMP1 in wildtype MEFs is strictly dependent on LMP1-induced protein synthesis. Further, these data indicate that LMP1 is capable of inducing the PI3K/Akt pathway and the ERK pathway by two different mechanisms. An initial, moderate wave of Akt and ERK activation is achieved by direct signaling mechanisms. The secondary, more prominent activation relies on secondary mechanisms and the synthesis of unknown proteins, which are needed for the activation of these pathways, possibly by autocrine or paracrine mechanisms.

4.4.2 LMP1-Dependent Production of Soluble Factors Induces Indirect Activation of STAT3, Akt and ERK

Since LMP1 failed to induce the activation of STAT3 and was hampered to fully activate Akt and ERK when protein synthesis was blocked, the question arose whether LMP1-dependent signaling was accountable for the induction of soluble factors, which could activate the three pathways in an autocrine/paracrine fashion. Therefore, MEFs were treated with conditioned medium harvested from wt MEF:NGFR-LMP1wt cells, which had been stimulated by antibody crosslinking for 3 or 6 hours. The 3 hour time point reflects the maximum activation of Akt and ERK by LMP1. The long 6 hour stimulation period was chosen to enrich factors in the supernatant at higher concentrations, because with prolonged NGFR-LMP1 signaling the soluble factors should accumulate in the medium. Wildtype as well as TRAF6$^{-/-}$ MEFs were used as target cells for the stimulation with conditioned medium. TRAF6$^{-/-}$ cells were chosen because crosslinking of NGFR-LMP1wt in TRAF6$^{-/-}$ MEFs did not result in any phosphorylation of STAT3, Akt or ERK (figure 4-13D, 4-14D and 4-15D). Since all three pathways are activated by indirect mechanisms, it was interesting to learn whether the lack of TRAF6 blocks the direct signaling pathways leading to the induction of soluble factors, or if TRAF6 is rather involved in the secondary signal transduction cascades induced by such factors. This was investigated by using both wildtype and TRAF6$^{-/-}$ MEFs as target cells (figure4-21).

Massive phosphorylation of STAT3 occurred when wildtype MEFs were treated with the conditioned supernatants from wt MEF:NGFR-LMP1wt cells that had been stimulated by antibody crosslinking for 3 or 6 hours (figure 4-21A). No activation of STAT3 occurred when supernatant from unstimulated cells (0 h) was used. This shows that LMP1-dependent signaling leads to the release of soluble factors, which are capable of activating STAT3 in the target cells. The supernatants from both the 3 hours and 6 hours stimulations were equally effective in inducing STAT3 phosphorylation. It is possible that STAT3 activation could not be further increased in response to the soluble factors and that the observed STAT3 phosphorylation represents the maximal phosphorylation possible.

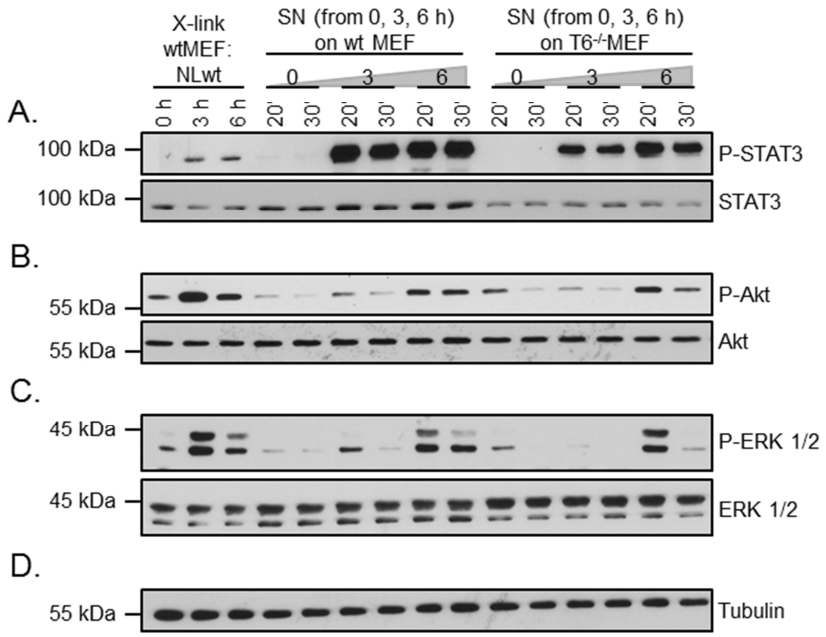

Figure 4-21. NGFR-LMP1-crosslinking induces the production of soluble factors that activate phosphorylation of STAT3, Akt and ERK in a mechanism that is independent of TRAF6. Wildtype MEFs stably expressing NGFR-LMP1wt (NLwt) were stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C and subsequent crosslinking (X-link) with 10 µg/ml α-fc IgG/IgM at 37°C for 3 h and 6 h. The supernatant (SN) was harvested and used to treat wildtype and TRAF6$^{-/-}$ MEFs for 20 min or 30 min, respectively. To analyze the activation of the STAT3, the PI3K/Akt and the ERK pathways, immunoblots for STAT3 phosphorylated at Tyr705 (**A.**), Akt phosphorylated at Ser473 (**B.**) and phosphorylation of ERK1/2 at Thr202 and Tyr204 (**C.**) were performed. Tubulin served as a loading control (**D.**).

Similarly, Akt was activated by soluble factors, which were released upon LMP1-crosslinking. Conditioned supernatant (3 h and 6 h) induced phosphorylation of Akt in the target wildtype MEFs compared to medium from unstimulated cells (figure 4-21B). Activation of Akt, however, was not as strong as STAT3 activation. It also seemed to depend more on a higher concentration of soluble factors in the supernatant, because supernatant harvested after 6 hours of stimulation was more potent in activating Akt than supernatant harvested after 3 hours, which induced Akt phosphorylation only mildly. The same was true for the activation of ERK (figure 4-21C). Conditioned medium collected after 6 hours of NGFR-LMP1-crosslinking induced ERK phosphorylation in the target cells, while the concentration of soluble factors after 3 hours was apparently not high enough to thoroughly activate ERK. In summary, the results demonstrate that both Akt and ERK are activated by LMP1 in an indirect manner through soluble factors released into the medium.

Using TRAF6$^{-/-}$ MEFs as target cells for the stimulation with conditioned supernatant demonstrated that TRAF6 is not essential for the secondary signaling pathways induced by soluble factors. STAT3, Akt and ERK were all activated upon treatment with conditioned medium in levels comparable to the activation in wildtype MEFs (figure 4-21). STAT3 was activated to a slightly lesser extent by soluble factors in TRAF6$^{-/-}$ MEFs, but this might be accountable to the overall lower levels of total STAT3 in these cells compared to wildtype MEFs. These results demonstrate also that TRAF6$^{-/-}$ MEFs are not generally defective with regard to the activation of STAT3, Akt and ERK.

4.4.3 CTAR2 and TRAF6 Are Essential for the Induction of Soluble Factors by LMP1

To confirm that LMP1 is unable to induce the production of soluble factors when CTAR2 is mutated or TRAF6 is missing, the conditioned supernatants of stimulated wt MEF:NGFR-LMP1(Y_{384}G) or TRAF6$^{-/-}$ MEF:NGFR-LMP1wt cells were tested for their ability to induce activation of STAT3, Akt and ERK in untreated wildtype MEFs. The assay was carried out in parallel to the one described in chapter 4.4.2, but in this case only wildtype MEFs were used as target cells. A control experiment with wt MEF:NGFR-LMP1wt cells as shown in figure 4-21 was performed next to both experiments shown in figure 4-22.

No increased activation of STAT3, Akt or ERK was observed after stimulation of wildtype MEFs with the conditioned supernatant from wt MEF:NGFR-LMP1(Y_{384}G) or TRAF6$^{-/-}$ MEF:NGFR-LMP1wt cells (figure 4-22A - C). This clearly demonstrates that LMP1 induces the release of soluble factors, which induce the activation of STAT3, Akt or ERK, via a CTAR2- and TRAF6-dependent pathway.

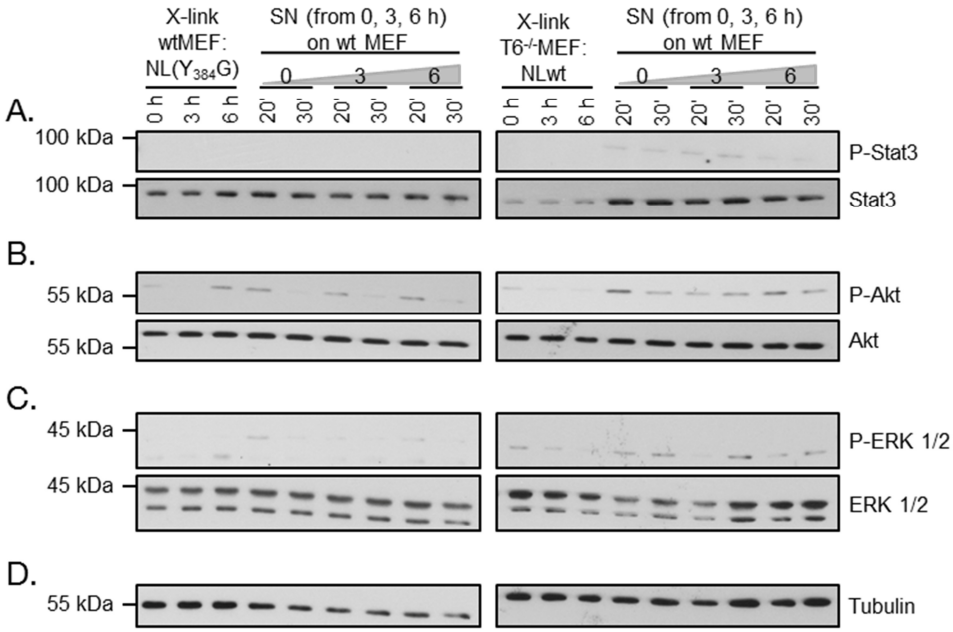

Figure 4-22. CTAR2 and TRAF6 are essential for the LMP1-dependent production of soluble factors that activate STAT3, Akt and ERK in an autocrine/paracrine manner. Wildtype MEFs stably expressing NGFR-LMP1($Y_{384}G$) (NL($Y_{384}G$)) and $TRAF6^{-/-}$ MEFs stably expressing NGFR-LMP1wt (NLwt) were incubated with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinked (X-link) with 10 µg/ml α-fc IgG/IgM at 37°C for 3 h and 6 h. Fresh wildtype MEFs were treated with the conditioned supernatants (SN) from the crosslinking kinetics for 20 min or 30 min. To analyze the activation of the STAT3, the PI3K/Akt and the ERK pathways, immunoblots for STAT3 phosphorylated at Tyr705 (**A.**), Akt phosphorylated at Ser473 (**B.**) and phosphorylation of ERK1/2 at Thr202 and Tyr204 (**C.**) were performed. Tubulin served as a loading control (**D.**).

4.4.4 LMP1 Is Responsible for the Upregulation of a Cocktail of Cytokines and Growth Factors

Activation of STAT3 by NGFR-LMP1 in MEFs is solely induced by indirect mechanisms, and the induction of the PI3K/Akt and ERK pathways is greatly enhanced through indirect signaling. Consequently, it was of great interest to identify the soluble factors that are induced by LMP1 signaling, and ultimately, which of them are responsible for the activation of STAT3, Akt and ERK. A number of studies have already demonstrated the upregulation of cytokines by LMP1, but available studies have only reported on a selected panel of factors, and a comprehensive analysis is lacking (Chew et al, 2010; Eliopoulos et al, 1999b; Hannigan et al, 2011; Ho et al, 1999; Li et al, 2007; Morris et al, 2008). Therefore I analyzed the NGFR-LMP1-dependent induction of cytokine and growth factors in MEFs by using a qRT-PCR (quantitative real time PCR) based screening array. For this the Qiagen "RT^2 Profiler" PCR Array was chosen, which included primers for 84 selected mouse growth factors and cytokines (listed in appendix 7.3).

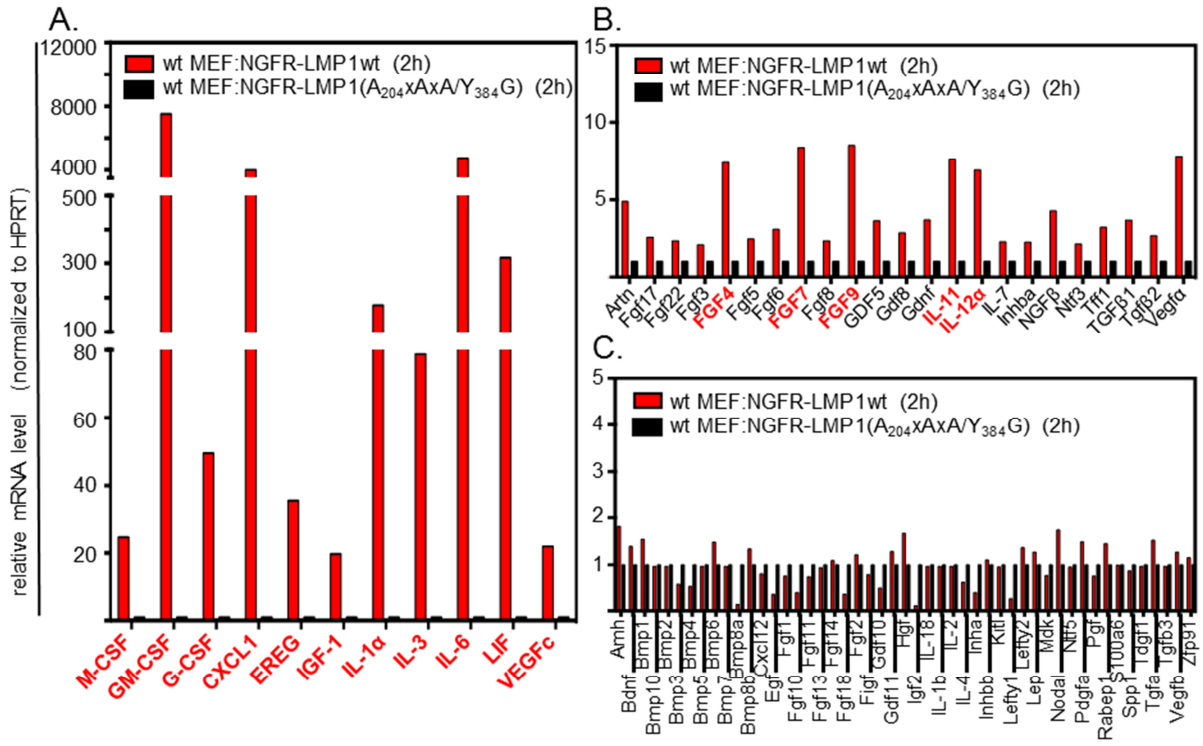

Figure 4-23. qRT-PCR array analysis identifies a cocktail of growth factors and cytokines that are upregulated immediately upon NGFR-LMP1 crosslinking. qRT-PCR array analysis of NGFR-LMP1-crosslinking in wildtype MEFs stably expressing NGFR-LMP1wt and (A_{204}xAxA/Y_{384}G). The cells were stimulated by staining with 1 µg/ml α-NGFR for 1 h at 37°C and crosslinking with 10 µg/ml IgG/IgM at 37°C for 2 h. The RNA was isolated and transcribed to cDNA, which was then used in a Qiagen RT^2 Profiler Array. The x-fold induction of mRNA by NGFR-LMP1wt is shown relative to a 1-fold base induction by NGFR-LMP1(A_{204}xAxA/Y_{384}G). The cytokines were distributed into groups according to their induction levels: more than 20-fold (**A.**), between 2 and 20-fold (**B.**) and non-induced or below 2-fold (**C.**). The cytokines and growth factors used for further analysis are written in red letters. The array was performed once.

To specifically identify primary targets of NGFR-LMP1wt, cDNA was generated from total RNA after 2 hours of stimulation of wt MEF:NGFR-LMP1wt cells. Direct NGFR-LMP1 signaling was detectable 30 to 60 minutes after crosslinking (see chapter 4-2). Therefore, mRNA extracted after 2 hours of crosslinking should mostly include the direct targets of these early signaling pathways, but not of later, indirect signaling pathways. At the same time, signaling after 2 hours should be advanced enough to yield enough specifically upregulated mRNA that can be detected in a qRT-PCR assay. wt MEF:NGFR-LMP1(A_{204}xAxA/Y_{384}G) cells served as a negative control for the array.

Figures 4-23A, B and C show the results of the qRT-PCR array. The results were split into three groups. Group 1 contains any factors that were induced above 20-fold in wt MEF:NGFR-LMP1wt

cells compared to wt MEF:NGFR-LMP1(A_{204}xAxA/Y_{384}G) (figure 4-23A). Group 2 includes all factors induced between 2-fold and 20-fold (figure 4-23B) and group three includes all factors that were either induced below 2-fold or were not induced (figure 4-23C). Among the hits were some that had already been published to be induced by LMP1 like IL-6 (Eliopoulos et al, 1997) or GM-CSF (Morris et al, 2008). This confirmed that the array was working properly.

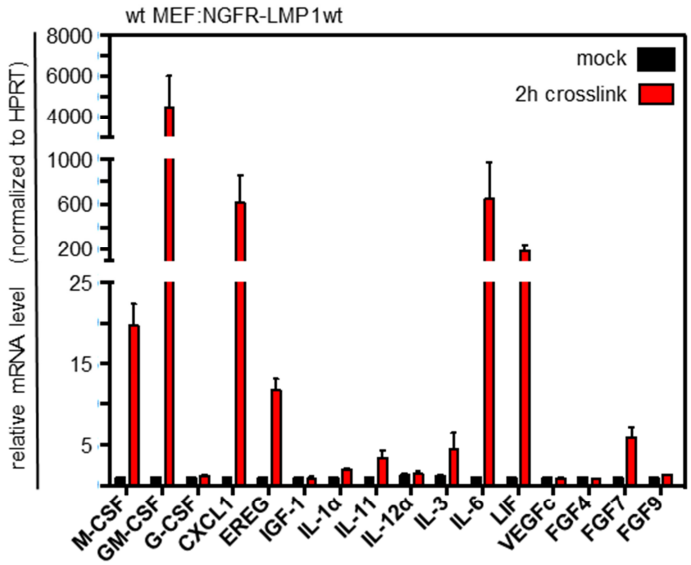

Figure 4-24. Verification of the array results by qRT-PCR. To verify and reproduce the results of the q RT-PCR array, selected cytokines and growth factors were analyzed in further qRT-PCR experiments. wt MEF:NGFR-LMP1wt cells were stimulated by NGFR-crosslinking for 2 h (1 µg/ml α-NGFR for 1 h at 37°C and 10 µg/ml α-fc IgG/IgM for 2 h at 37°C), and RNA was isolated, transcribed to cDNA and used for qRT-PCR assays with the primers for the cytokines and growth factors indicated. The relative x-fold induction levels of the indicated factors are shown in comparison between mock and 2 h crosslink of 3 independent experiments. Error bars represent SD.

Due to its high costs, the array was only performed once. Therefore, further verification of the positive results was mandatory. Only a reduced panel of targets was extensively studied in detail. All hits below 5-fold induction were not prioritized for further analysis. Those cytokines and growth factors that were analyzed in further detail are written in red letters in figure 4-23A and B and include M-CSF, GM-CSF, G-CSF, CXCl1, EREG, IGF-1, IL-1α, IL-11, IL-12α, IL-3, IL-6, LIF, VEGFc, FGF4, FGF7 and FGF9. To verify the induction of these cytokines and growth factors in an independent assay, qRT-PCRs were conducted using cDNA generated from wt MEF:NGFR-LMP1wt cells after 2 hours of NGFR-LMP1 stimulation or without stimulation as a negative control. Thereby, it was possible to examine the definite induction of cytokines and

growth factors upon NGFR-LMP1 crosslinking in comparison to unstimulated cells. Figure 4-24 summarizes the results of three independent experiments. The results showed that some of the data from the array could be reproduced, but some hits from the primary array appeared to be false positive hits. G-CSF, IGF-1, IL-12α, VEGFc, FGF4 and FGF9 were not induced after 2 hours of crosslinking compared to the unstimulated control. However, massive induction of GM-CSF, CXCL1, IL-6 and LIF in NGFR-LMP1wt-stimulated cells was confirmed. Induction of GM-CSF was highest with an average fold-induction of above 4000 compared to the unstimulated control. CXCL1 and IL-6 were induced about 615-fold and 650-fold, respectively, while LIF was induced 193-fold on average. M-CSF (20-fold), EREG (12-fold) and FGF7 (6-fold) were also significantly upregulated after 2 hours of stimulation. Upregulation of LIF was particularly interesting, because LIF has never before been shown to be a direct target of LMP1.

Taken together, the qRT-PCR screen and the subsequent follow-up analysis of the data demonstrate that NGFR-LMP1wt is capable of upregulating a cocktail of cytokines and growth factors upon crosslinking-stimulation. Some of these, including IL-6, GM.CSF, EREG or CXCL1 (Charalambous et al, 2007; Eliopoulos et al, 1997; Hannigan et al, 2011; Morris et al, 2008), confirm already published data, demonstrating that the assay and the system work as expected. However, LIF is a new direct target of LMP1 and is particularly interesting, because it is not only involved in cancer-related inflammation, but also known to induce STAT3, Akt and ERK in different contexts (Magni et al, 2007; Silver & Hunter, 2010; Slaets et al, 2008).

4.4.5 CTAR2 and TRAF6 Are Critically Involved in the Upregulation of Inflammatory Cytokines and Growth Factors by LMP1

In this study it was shown that lack of TRAF6 or mutations crippling CTAR2 render NGFR-LMP1 incapable of inducing activation of STAT3, Akt and ERK through soluble factors. This led to the question if the upregulation of the pro-inflammatory cytokines and growth factors obtained from the qRT-PCR array was also depending on CTAR2 and TRAF6. Therefore wt MEF:NGFR-LMP1($Y_{384}G$) and $TRAF6^{-/-}$ MEF:NGFR-LMP1wt cells were tested by qRT-PCR for the induction of selected factors. Only those cytokines and growth factors which were significantly enhanced after 2 hour crosslinking of wt MEF:NGFR-LMP1wt cells were tested, and G-CSF was included as a negative control.

The cell lines wt MEF:NGFR-LMP1wt, wt MEF:NGFR-LMP1($Y_{384}G$) and $TRAF6^{-/-}$ MEF:NGFR-LMP1wt were stimulated for 2 hours by antibody-crosslinking or left untreated, before total mRNA was extracted to generate cDNA and perform qRT-PCR with primers for M-CSF, GM-CSF, G-CSF, CXCL1, EREG, IL-6, LIF and FGF7. Stimulation of NGFR-LMP1wt upregulated all factors apart from G-CSF with high statistical significance ($p < 0.001$,

figure 4-25). However, neither the CTAR2 mutant nor NGFR-LMP1wt in TRAF6$^{-/-}$ MEFs were capable of inducing any of the tested mRNAs. This shows that LMP1 signaling events originating at CTAR2 and relying on TRAF6 are crucial for the upregulation of all examined cytokine and growth factor mRNAs. This also supports the role of CTAR2-dependent signaling in the autocrine/paracrine stimulation of signaling pathways.

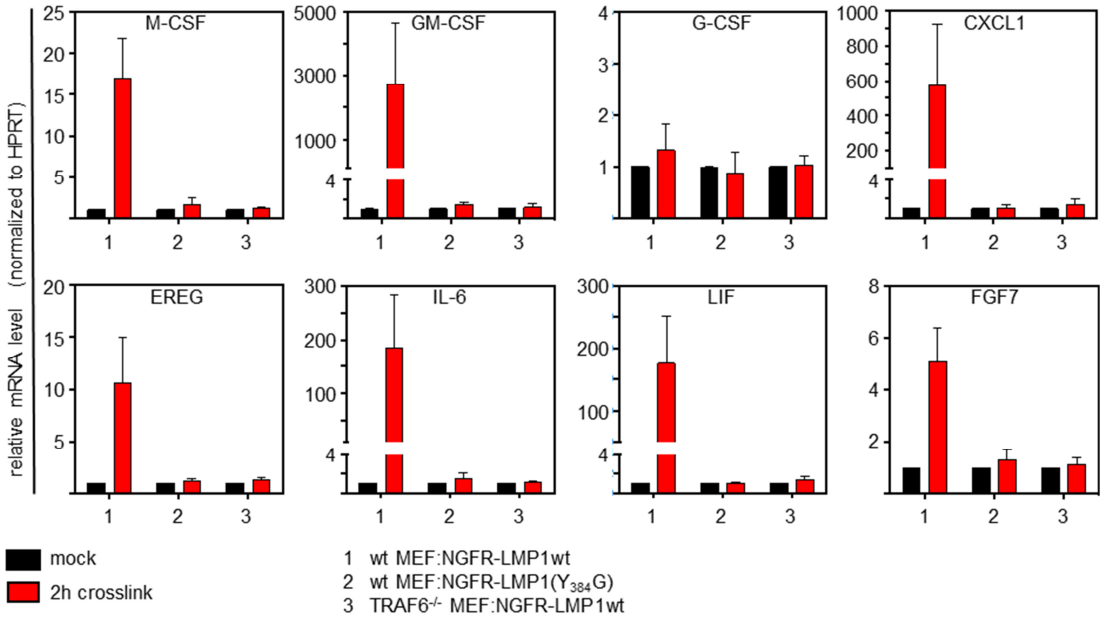

Figure 4-25. A functional CTAR2 domain and TRAF6 are required for the upregulation of distinct cytokines and growth factors by NGFR-LMP1. The cell lines wt MEF:NGFR-LMP1wt, wt MEF:NGFR-LMP1($Y_{384}G$) and TRAF6$^{-/-}$ MEF:NGFR-LMP1wt were stimulated by NGFR-crosslinking for 2 h (1 µg/ml α-NGFR for 1 h at 37°C and 10 µg/ml α-fc IgG/IgM at 37°C for 2 h), and the extracted RNA was transcribed to cDNA to be used in qRT-PCR experiments using primers for the indicated cytokines and growth factors. G-CSF served as a negative control. Induction values of all tested mRNAs after 2 h compared to unstimulated controls (mock) of wt MEF:NGFR-LMP1wt (except G-CSF) were highly significant in a non-paired heteroscedastic t-test ($p < 0.001$). The data were combined from 10 independent experiments in the case of wt MEF:NGFR-LMP1wt or 3 independent experiments in the case of wt MEF:NGFR-LMP1($Y_{384}G$) and TRAF6$^{-/-}$ MEF:NGFR-LMP1wt. Error bars represent SD.

4.4.6 LIF and GM-CSF Are the Major Inducers of Indirect Activation of STAT3, Akt and ERK by LMP1

Activation of STAT3 and partly of Akt and ERK by LMP1 depends on the release of soluble factors, and LMP1-dependent signaling is capable of upregulating a cocktail of inflammatory cytokines and growth factors. Consequently, the question arose, which of the found factors was

specifically responsible for the activation of STAT3, Akt and ERK. To test this, antagonistic antibodies were used to neutralize the soluble factors in conditioned medium from wt MEF:NGFR-LMP1wt cells stimulated by antibody-crosslinking. Conditioned supernatant was harvested after 3 and 6 hours of stimulation and from unstimulated cells to be used for restimulation of wildtype MEFs. Prior to restimulation aliquots of this supernatant were incubated with neutralizing antibodies against LIF, IL-6, CXCL1, EREG or M-CSF, or left untreated. GM-CSF was neutralized by incubating the target cells directly with neutralizing antibodies directed against the GM-CSF receptor. Neutralizing antibodies were used at a concentration of 10 µg/ml. This concentration was chosen because it exceeded the neutralization dose (ND_{50}) for recombinant proteins specified by the manufacturer between 2- and 100-fold. Also, using higher concentrations of neutralizing antibody (20 µg/ml) did not increase the effect of neutralization (data not shown). After preincubation the supernatants were used to stimulate wildtype MEFs for 20 minutes, and the activation of STAT3, Akt and ERK was examined on immunoblots.

Neutralization of LIF almost completely suppressed the activation of STAT3 by conditioned supernatant (figure 4-26A, lanes 8, 9). No significant effect on STAT3 activation was observed after neutralization of any other factor (figure 4-26A, lanes 11, 12 and figure 4-26E). Interestingly, STAT3 phosphorylation was only very slightly decreased by neutralization of IL-6, and this effect could not be increased by raising the concentration of neutralizing antibodies (figure 4-26E, lane 18, and data not shown). This was unexpected since it was reported that IL-6 is predominantly responsible for STAT3 activation in epithelial cells (Chen et al, 2003). Neutralization of either LIF or GM-CSF reduced the phosphorylation of Akt by conditioned medium, and neutralization of both LIF and GM-CSF at the same time (“mix”) almost completely abrogated the activation of Akt (figure 4-26B). This indicates that LIF and GM-CSF cooperativley induce the PI3K/Akt pathway. Neutralization of EREG had only a very faint effect on Akt phosphorylation (figure 4-26F, lanes 14, 15), and neither M-CSF, CXCL1 nor IL-6 seemed to be involved in activating Akt (figure 4-26F). ERK activation was also mostly dependent on LIF and GM-CSF, although the contribution of LIF was greater. Neutralization of both factors alone reduced phosphorylated ERK in the target cells (figure 4-26, lanes 8, 9 and lanes 11, 12), which was further reduced to almost nothing by mixing both antibodies for neutralization (figure 4-26, lanes 14, 15). None of the other neutralizing antibodies visibly affected ERK phosphorylation (figure 4-26G).

Taken together, this result demonstrates that in MEFs the LMP1-dependent indirect activation of Akt and ERK depends primarily on LIF and GM-CSF combined, while LIF is almost exclusively essential for STAT3 activation, with a possible, small contribution of IL-6.

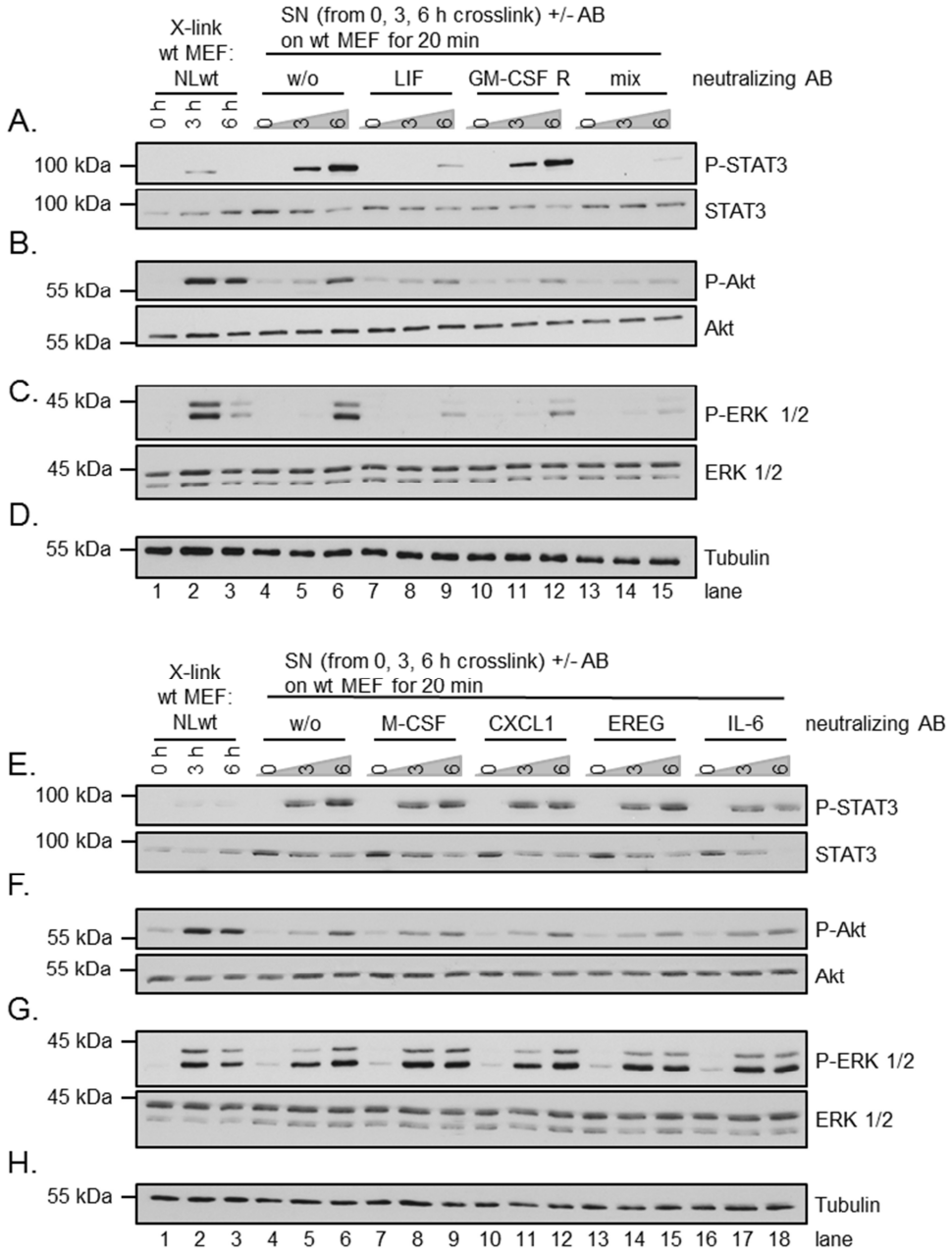

Figure 4-26. Soluble factors in the supernatant of wt MEF:NGFR-LMP1wt stimulated by NGFR-crosslinking can be neutralized by antibodies. Wildtype MEFs stably expressing NGFR-LMP1wt (NLwt) were stimulated by crosslinking (X-link) NGFR-LMP1 (1 µg/ml α-NGFR for 1 h at 37°C followed by 10 µg/ml α-fc IgG/IgM at 37°C) for 3 h and 6 h. Wildtype MEFs were treated with the supernatants (SN) from the crosslinking kinetics for 20 min, after pre-incubating the SN with or without 10 µg/ml neutralizing antibodies against the indicated soluble factors for 1 h. GM-CSF was neutralized by incubating the wildtype MEF target cells with 10 µg/ml neutralizing antibody against the GM-CSF receptor for 1 h. To analyze the activation of the STAT3, the PI3K/Akt and the ERK pathways, immunoblots for STAT3 phosphorylated at Tyr705 (**A.** and **E.**), Akt phosphorylated at Ser473 (**B.** and **F.**) and phosphorylation of ERK1/2 at Thr202 and Tyr204 (**C.** and **G.**) were performed. Tubulin served as a loading control (**D.** and **H.**).

4.4.7 CTAR2-Induced Signaling Pathways Are Responsible for the Upregulation of Selected Cytokines and Growth Factors by LMP1

The CTAR2 domain was critically involved in the upregulation of selected cytokines like LIF and GM-CSF, which were found to be major inducers of STAT3, Akt and ERK. This raised the question which of the CTAR2-dependent signaling pathways was specifically responsible for the upregulation of the tested cytokines and growth factors.

It was shown before that the canonical NF-κB pathway and the p38/MAPK pathway mediate the induction of IL-6 by LMP1 (Eliopoulos et al, 1999b; Eliopoulos et al, 1997), and the JNK pathway had been shown to be involved in the upregulation of IL-8 (Yoshizaki et al, 2001). All three pathways originate at CTAR2, as it was shown in chapter 4.2. Therefore, these pathways were inhibited by specific inhibitors, and the induction of specific mRNAs encoding M-CSF, GM-CSF, G-CSF, CXCL1, EREG, IL-6, LIF and FGF7 was analyzed by qRT-PCR after crosslinking of NGFR-LMP1wt. The JNK pathway was inhibited by SP600125 (Bennett et al, 2001), p38/MAPK was inhibited by SB203580 (Kumar et al, 1999), and JSH-23, which blocks the nuclear translocation of p65 (Shin et al, 2004), was used to block the canonical NF-κB pathway.

All three inhibitors tested had an effect on the upregulation of certain mRNAs, but with varying efficiencies (figure 4-27). Upregulation of IL-6 was blocked almost completely by all three inhibitors. Since it has already been published that both the canonical NF-κB pathway and the p38/MAPK pathway lead to the upregulation of IL-6 by LMP1, this result serves as a positive control for the assay. Notably, the JNK inhibitor SP600125 blocked upregulation of IL-6 just as potently as the other two inhibitors. At the same time, SP600125 and SB203580 did not significantly reduce the upregulation of FGF7 mRNA. Similarly, inhibition of JNK had no negative effect on the induction of CXCL1 mRNA. These results may serve as a negative control for the experiment. The observed differences also demonstrate that the inhibitory effects were specific, and the tested inhibitors did not globally downregulate the mRNA levels by off-target effects.

Importantly, the induction of LIF, which had turned out to be the major inducer of STAT3 in LMP1-dependent signaling, was diminished to about 20 % of the untreated control by inhibition of JNK or NF-κB. The p38 inhibitor SB203580 had a comparable effect on LIF mRNA and reduced the levels to an average of 30 % of the control. Similarly, GM-CSF, which was also involved in the activation of Akt and ERK, was not fully induced in the presence of JSH-23 or SP600125. While the p38/MAPK inhibitor SB203580 had only a mild, insignificant effect on the upregulation of GM-CSF mRNA, inhibition of the JNK and canonical NF-κB pathway reduced the relative GM-CSF mRNA levels to approximately 40 % and 20 % of the maximum induction, respectively. These results lead to the conclution that the CTAR2-dependent canonical NF-κB,

the JNK and the p38/MAPK pathways cooperate to induce the upregulation of LIF and GM-CSF in order to trigger the indirect activation of STAT3, Akt and ERK.

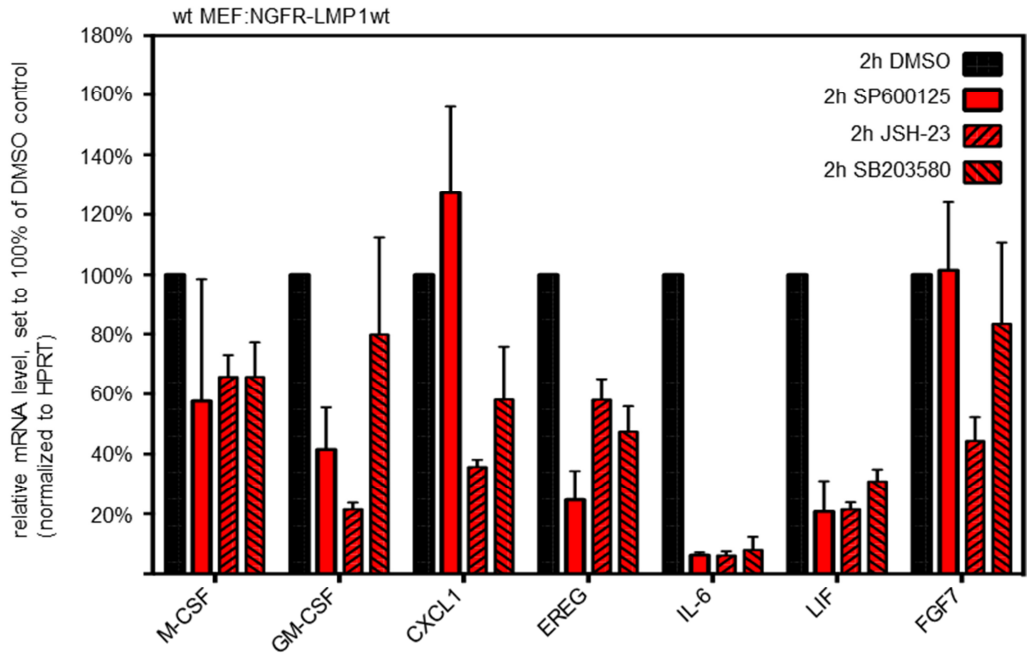

Figure 4-27. Inhibition of the JNK pathway, the canonical NF-κB pathway and the p38/MAPK pathway during NGFR-LMP1-crosslinking affects the upregulation of CTAR2-induced cytokines and growth factors. wt MEF:NGFR-LMP1wt cells were stimulated by NGFR-crosslinking for 2 h (1 µg/ml α-NGFR for 1 h at 37°C followed by 10 µg/ml α-fc IgG/IgM at 37°C for 2 h) in the presence or absence of inhibitors. The JNK pathway was inhibited with 10 µM SP600125, and the p38/MAPK pathway was inhibited with 1 µM SB203580. The cells were preincubated with both inhibitors for 1 h during α-NGFR binding and fresh inhibitor was added along with IgG/IgM. 30 µM JSH-23 was used to inhibit the canonical NF-κB pathway. This compound was only present during the crosslinking with IgG/IgM without preincubation. The extracted RNA was transcribed to cDNA to be used in qRT-PCR experiments using the primers for the indicated cytokines and growth factors. The induction levels of 2 h stimulation with DMSO relative to an unstimulated control (not shown) were set to 100%, and the percentage of induction in the presence of inhibitors was calculated in relation to that. Error bars represent the standard deviation.

Similarly, the upregulation of mRNAs for M-CSF, CXCL1, EREG and FGF7 were differentially influenced by the presence of the tested inhibitors. JSH-23 and SB203580 reproducably down-regulated the induction of M-CSF to about 60 %. On average, SP600125 had a smiliar effect on M-CSF, but this was not significant. CXCL1 mRNA levels were reduced to about 40 % and 60 % by JSH-23 and SB203580, respectively. As mentioned before, inhibition of JNK did not reduce CXCL1 mRNA levels. Upregulation of EREG mRNA was also partially reduced by all three inhibitors, with SP600125 having the greatest impact with about 80 % reduction. FGF7 was only

significantly reduced to 40 % by inhibition of the canonical NF-κB pathway, while inhibition of both MAPK pathways had no or little effect.

Taken together, these results demonstrate that different CTAR2- and TRAF6-dependent pathways contribute differently to the upregulation of cytokines and growth factors by LMP1. However, all three tested pathways are involved in the upregulation of LIF, which is the primary mediator of STAT3 activation by NGFR-LMP1 in MEFs and plays a role in the activation of Akt and ERK. At the same time the canonical NF-κB pathway and the JNK pathway cooperate to upregulate GM-CSF, which is also involved in the autocrine/paracrine activation of Akt and ERK by NGFR-LMP1wt in MEFs.

4.4.8 Inhibition of JNK, p38 MAPK and NF-κB Reduces the Activation of STAT3, Akt and ERK by NGFR-LMP1

The canonical NF-κB pathway, the JNK pathway and the p38/MAPK pathway were involved in the upregulation of cytokines and growth factors, which induce the indirect activation of STAT3, Akt and ERK. Therefore, the question remained which pathway was involved in the activation of STAT3, Akt and ERK. As a logical consequence, inhibition of the three pathways should have negative effects on STAT3, Akt and ERK activation. To test this, crosslinking experiments were conducted with wt MEF:NGFR-LMP1wt cells in the presence or absence of the inhibitors SP600125, JSH-23 and SB203580, and the results are summarized in figure 4-28.

Inhibition of the canonical NF-κB pathway and the JNK pathway by JSH-23 and SP600125, respectively, significantly reduced the ability of NGFR-LMP1wt to activate STAT3 (figure 4-28A). No phosphorylated STAT3 was observed at 90 minutes of crosslinking in the presence of both inhibitors, and phosphorylation was greatly diminished at 120 minutes. It must be noted that the STAT3 phosphorylation at the late 180 min time point was comparable regarding the inhibition of JNK or NF-κB and the untreated control. This hints at a delayed time course of STAT3 activation in the presence of both inhibitors. Inhibition of p38 MAPK by SB203580 also diminished the phosphorylation of STAT3 at 90 minutes, but the reduction was less obvious at later time points (figure 4-28A). It is likely that effective LIF levels are only reached at later time points, because LIF production is reduced in the presence of the inhibitors. Inhibition of p38 MAPK reduced LIF mRNA induction to about 30 % in contrast to 20 % in the presence of JSH-23 and SP600125 (figure 4-27). Therefore it is possible that this inhibitor only delays LIF production instead of reducing it to a level where STAT3 activation is significantly diminished. Taken together, these results complement the previously described results concernig LIF activity and regulation. LIF was the major inducer of STAT3 (chapter 4.4.6), and LIF mRNA levels were reduced in the presence of all three inhibitors, with more pronounced effects of SP600125 and JSH-23 (chapter

4.4.7). Combining these results with the inhibitory effects of JSH-23, SP600125 and SB203580 on STAT3 phosphorylation by NGF-LMP1 shown here, demonstrates that all three pathways cooperate with varying efficiencies to upregulate LIF (figure 4-27) in order to induce the activation of STAT3, or that overlapping mechanisms must be responsible for this.

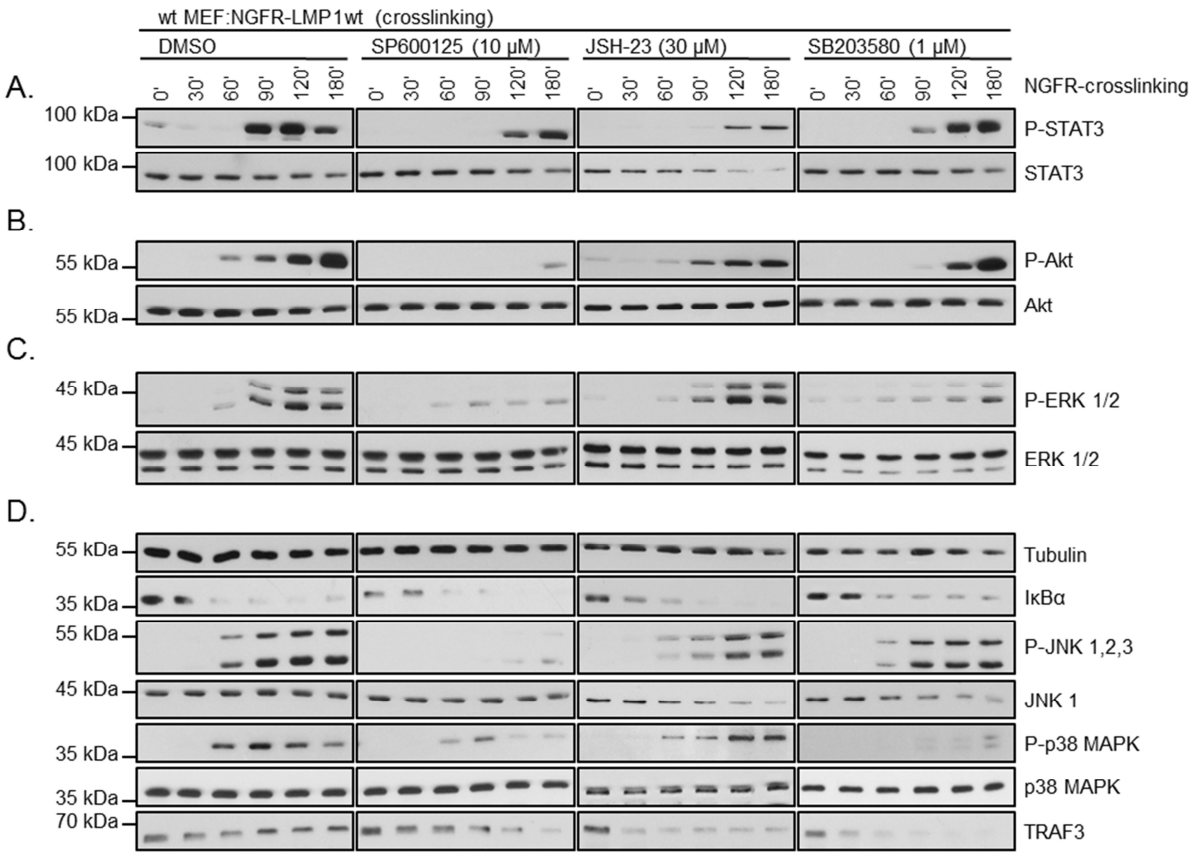

Figure 4-28. Inhibition of the JNK pathway, the canonical NF-κB pathway and the p38/MAPK pathway during NGFR-LMP1-crosslinking leads to a diminished phosphorylation of STAT3, Akt and ERK. Wildtype MEFs stably expressing NGFR-LMP1wt (NLwt) were stimulated by crosslinking (X-link) the surface NGFR with 1 µg/ml α-NGFR for 1 h at 37°C and 10 µg/ml α-fc IgG/IgM at 37°C for 30 min to 180 min in the presence or absence of inhibitors. The JNK pathway was inhibited with 10 µM SP600125, and the p38/MAPK pathway was inhibited with 1 µM SB203580. The cells were preincubated with both inhibitors for 1 h during α-NGFR binding and fresh inhibitor was added with α–fc IgG/IgM. 30 µM JSH-23 was used to inhibit the canonical NF-κB pathway. This compound was only present during the crosslinking with IgG/IgM without preincubation. To analyze the activation of the STAT3, the PI3K/Akt and the ERK pathways, immunoblots for STAT3 phosphorylated at Tyr705 (**A.**), Akt phosphorylated at Ser473 (**B.**) and phosphorylation of ERK1/2 at Thr202 and Tyr204 (**C.**) were performed. Tubulin served as a loading control, and the induction of the JNK pathway, the canonical NF-κB pathway, the p38/MAPK pathway and the non-canonical NF-κB pathway were analyzed to serve as a positive control for the inhibitors, but also to rule out any major side effects of the inhibitor treatment on the cells and the crosslinking kinetics of other pathways (**D.**).

Inhibition of JNK by SP600125 had the greatest impact on the activation of Akt by NGFR-LMP1, which was almost completely blocked by JNK inhibition (figure 4-28B). At the same time JSH-23 specifically reduced the maximum induction of Akt at 180 minutes of stimulation (figure 4-28B). This late Akt activation was mostly dependent on de novo synthesized factors (chapter 4.4.1), suggesting that NF-κB inhibition has a highly specific effect on the late activation of Akt. Inhibition of the p38/MAPK pathway, however, had only marginal effects on Akt activation (figure 4-28B). While all three inhibitors blocked LIF upregulation to a similar extent, GM-CSF mRNA was most effectively blocked by JSH-23 and less effectively by SP600125, while SB203580 had no significant effect (chapter 4.4.7). At the same time neutralization of GM-CSF or LIF were equally effective in partially blocking Akt activation (chapter 4.4.6). Taken together, these observations suggest that the p38/MAPK pathway is only partially involved in the indirect activation of Akt through LIF regulation. Canonical NF-κB and JNK, on the other hand, seem to employ overlapping or cooperative mechanisms that involve both GM-CSF and LIF upregulation. However, it is not clear why JSH-23 has a greater effect on blocking GM-CSF upregulation, while SP600125 was significantly more effective in blocking Akt activation. Possibly the JNK pathway additionally regulates other mechanisms that were not entirely covered by the experiments at hand, and that are responsible for the potent inhibition of Akt phosphorylation. These mechanisms might include a variety of factors. One of these factors could be EREG. Induction of this factor was most effectively blocked by JNK inhibition (chapter 4.4.7), and at the same time, neutralization of EREG had a slight effect on Akt activation by conditioned medium (chapter 4.4.6).

Activation of ERK was most influenced by the MAPK pathways. Inhibition of JNK reduced ERK phosphorylation to very low levels, and p38 inhibition significantly blocked activation of ERK. JSH-23, on the other hand, had no effect on the activation of ERK by NGFR-LMP1 (figure 4-28C). This suggests that only JNK and p38/MAPK cooperate to activate ERK in NGFR-LMP1 signaling. Activation of ERK by soluble factors was partially blocked by neutralization of GM-CSF and LIF, respectively, with a slightly greater effect of LIF neutralization. Both LIF and GM-CSF mRNA were more potently downregulated by SP600125 than SB203580, which reflects the greater impact of the JNK inhibitor on ERK activation. However, it is not clear why JSH-23, which potently reduced GM-CSF and LIF mRNA levels, had no effect on ERK activation. In fact, JSH-23 treatment rather slightly enhanced the phosphorylation of ERK after 180 minutes of crosslinking. Possibly, this strong ERK response represents an off-target response to treatment with JSH-23, which overrides any specific effect of the inhibtior on the indirect activation of ERK by NGFR-LMP1.

To test, whether any of the inhibitors induced side-effects on other pathways, which are directly activated by NGFR-LMP1, immunoblots were performed to visualize the activation of the canonical NF-B pathway, the JNK and p38/MAPK pathway and TRAF3 reduction as an effect of CTAR1 dependent signaling (figure 4-28D). As expected, none of the inhibitors had any major side-effect on other pathways induced by NGFR-LMP1. Degradation of IκBα was induced with comparable kinetics in all samples, and JNK phosphorylation was inhibited by SP600125, but not by JSH-23 or SB203580. Only a small reduction of p38 phosphorylation by the JNK-inhibitor SP600125 was evident (figure 4-28D). This is a known side-effect of SB203580 (Clerk & Sugden, 1998), and the low concentration of SB203580 (1 µM) was chosen in order to minimize that problem.

Taken together the presented data in the present and the previous two chapters demonstrate that inhibition of the JNK pathway, the p38/MAPK pathway and the canoncial NF-κB pathway blocks the activation of STAT3, Akt and ERK via LIF and GM-CSF with differential efficiencies. Cooperative or overlaying mechanisms are likely responsible for these indirect activation loops. It is also evident that CTAR2-dependent signaling mechanisms are responsible for the induction of a wide varitey of signaling pathways both by direct and indirect mechanisms.

4.4.9 LMP1 Induces Cytokines and Growth Factors in a CTAR2-Dependent Manner in CNE-L Cells, and STAT3 Is Activated by LMP1 by an Indirect Mechanism

The previous chapters demonstrated that inducible NGFR-LMP1 is capable of upregulating selected cytokines and growth factors in a CTAR2- and TRAF6-dependent manner in MEFs. Thereby, STAT3, Akt and ERK are induced in an autocrine/paracrine fashion. Since MEFs are not a natural target of EBV, it was mandatory to investigate whether similar mechanisms of LMP1 signaling exist in cells that are natural targets of EBV. For this purpose, the EBV-negative human nasopharyngeal carcinoma cell line CNE-L was chosen, because LMP1 expression is a hallmark of EBV-associated NPC, and because it was shown that dysregulated STAT3 is often associated with malignant NPC (Ho et al, 2013; Liu et al, 2008; Lui et al, 2009a).

First, the ability of HA-tagged native wildtype LMP1 to induce a selection of cytokines and growth factors in CNE-L cells was tested. This selection was based on the factors found to be upregulated by NGFR-LMP1wt in MEFs (chapter 4.4.4 and 4.4.5). Therefore, CNE-L cells were transiently transfected with HA-LMP1wt, HA-LMP1($Y_{384}G$) or an empty control for 24 hours, before the relative induction of mRNAs encoding for M-CSF, GM-CSF, G-CSF, CXCL1, EREG, IL-6, LIF and FGF7 was analyzed by qRT-PCR. LMP1 induced a panel of factors in CNE-L cells, which was not identical with but very similar to that induced in MEFs, including M-CSF, CXCL1, EREG, IL-6 and LIF (figure 4-29A). The mRNA levels for all these factors were drastically

reduced when the CTAR2 domain was mutated (figure 4-29A). This demonstrates that CTAR2-dependent signaling pathways are responsible for the upregulation of the tested cytokines and growth factors in CNE-L cells as well. However, the overall relative mRNA expression levels were much lower in CNE-L cells than in MEFs, which can likely be attributed to the steady-state signaling that is to be expected upon expression of constitutively active LMP1 after 24 h. In contrast, no GM-CSF or FGF7 was induced after expression of HA-LMP1wt in CNE-L compared to the negative control. This was especially surprising with regard to GM-CSF, because this cytokine was massively induced in MEFs, and it played a role in the indirect activation of Akt and ERK in these cells. Additionally, the results demonstrate that native LMP1 signaling drives cellular responses similar to inducible NGFR-LMP1.

The next question was whether CTAR2-dependent signaling was also essential for the release of soluble factors, and with that for the activation of STAT3, Akt and ERK in CNE-L cells. To test this scenario, the conditioned supernatant from CNE-L cells, which were transiently transfected with HA-tagged LMP1wt, LMP1(Y_{384}G) or LMP1(A_{204}xAxA/Y384G) for 24 hours, was harvested and used to stimulate CNE-L cells for 20 or 30 minutes. The activation of STAT3, Akt and ERK was assessed by immunoblotting (figure 4-29B). It was important to starve the CNE-L target cells in serum-free medium before stimulation, because the cells displayed very high levels of constitutively phosphoralyted Akt in the presence of FCS (data not shown).

Medium conditioned by HA-LMP1wt induced phosphorylation of STAT3 in CNE-L cells. In contrast, no activation of STAT3 occurred when supernatant conditioned by LMP1(Y_{384}G) or LMP1(A_{204}xAxA/Y_{384}G) was used (figure 4-29B). This result strengthens the point that CTAR2-dependent upregulation of soluble factors is essential for autocrine/paracrine activation of STAT3 in nasopharyngeal carcinoma cell lines. However, the overall strength of STAT3 phosphorylation was much weaker in CNE-L cells than in MEFs. This could be accountable to the weaker upregulation of inflammatory factors by native LMP1 in CNE-L compared to inducible NGFR-LMP1 in MEFs. It is also possible that the induction of soluble factors and their efficiency in restimulation experiments are much stronger with a distinct starting point of receptor activation, which was the case for NGFR-LMP1, but not for native HA-LMP1.

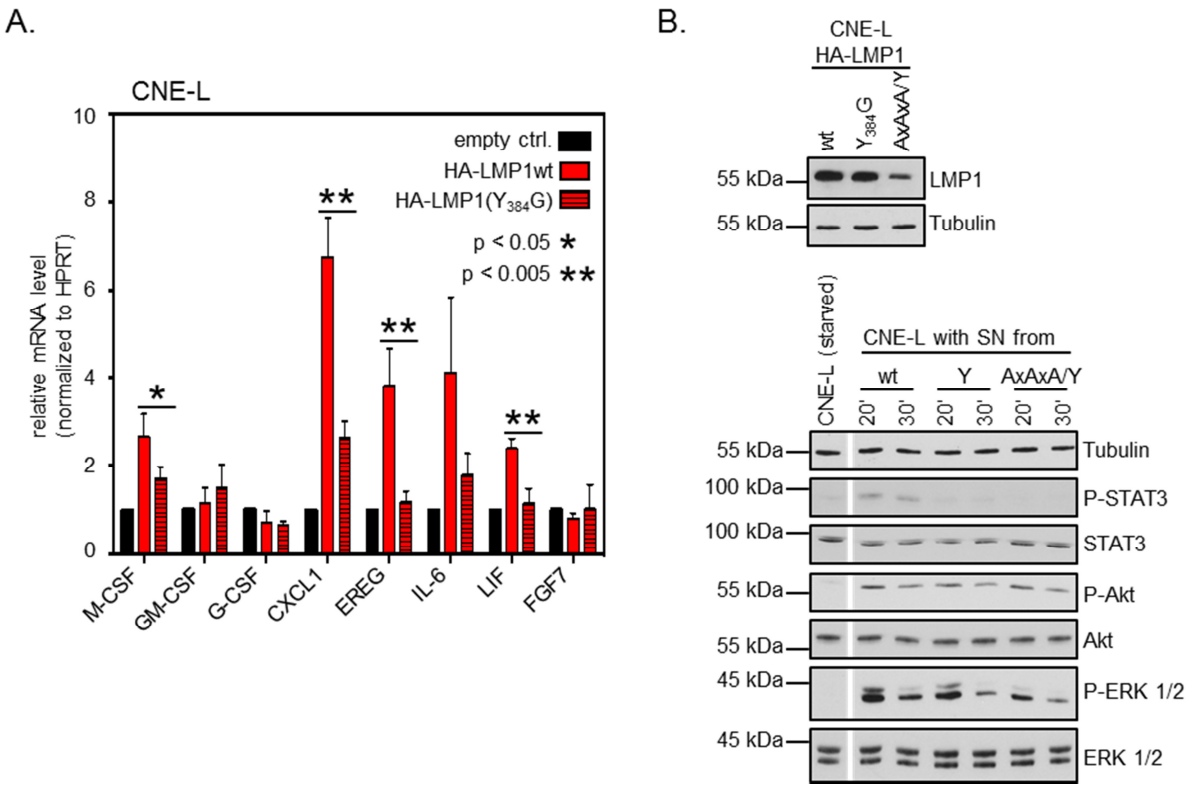

Figure 4-29. LMP1-CTAR2 induces distinct cytokines and growth factors and the indirect activation of STAT3 in the nasopharyngeal carcinoma cell line CNE-L. A. CNE-L cells were transfected with 1 µg expression plasmids pSV-HA-LMP1wt or pSV-HA-LMP1($Y_{384}G$) per 5 x 10^5 cells for 24 h. Total RNA was extracted to be transcribed to cDNA and used for qRT-PCR with the primers for the indicated growth factors and cytokines. The induction levels were calculated relative to an empty transfection control, and a non-paired heteroscedastic t-test was used to determine the significance of the difference between the fold induction of LMP1wt and LMP1($Y_{384}G$). The results represent 4 independent experiments. **B**. CNE-L cells were transfected with expression plasmids pSV-HA-LMP1wt (wt) or pSV-HA-LMP1($Y_{384}G$) (Y) and pSV-HA-LMP1($A_{204}xAxA/Y_{384}G$) (AxAxA/Y) and starved in FCS-free medium for 24 h (1 µg plasmid per 5 x 10^5 cells), before the supernatant (SN) was collected and fresh CNE-L cells, which had also been starved in serum-free medium before stimulation, were incubated with the supernatant for 20 and 30 min. LMP1 expression in the transfected cells was shown by immunoblotting with α–LMP1 3G6-1 (upper paenel). To analyze the activation of the STAT3, the PI3K/Akt and the ERK pathways, immunoblots for STAT3 phosphorylated at Tyr705, Akt phosphorylated at Ser473 and phosphorylation of ERK1/2 at Thr202 and Tyr204 were performed (lower panel). Tubulin served as a loading control.

The activation of Akt with conditioned medium in CNE-L cells unfortunately proved difficult to evaluate. Akt was activated by all supernatants tested, with no differences regarding LMP1wt or mutant. Even supernatant from cells transfected with the signaling-inactive mutant LMP1($A_{204}xAxA/Y_{384}G$) induced Akt phosphorylation similarly to supernatant from LMP1wt-transfected cells. This suggests that the supernatant of CNE-L cells contains a factor independent of LMP1 expression that can activate Akt, and triggering of the PI3K/Akt pathway

by this means was not exclusively dependent on a specific LMP1-related mechanism. Instead it is possible that the transfection procedure itself resulted in the production of an unknown factor that activated Akt, or that the phosphorylation of Akt represents a stress response to the stimulation procedure. Since CNE-L cells are a transformed carcinoma cell line, it is also likely that they constantly produce soluble factors, which activate Akt via autocrine/paracrine loops. These assumptions are supported by the fact that CNE-L cells have constitutively high levels of phosphorylated Akt as long as they are not starved in FCS-free medium, which suggests that these cells are highly sensitive to Akt activation (data not shown).

Phosphorylation of ERK was also induced by all three supernatants in question. However, here it seemed that phosphorylation was strongest in the cells treated with supernatant conditioned by LMP1wt. Nevertheless, an exclusive induction by medium harvested from LMP1wt-transfected cells was not observed. Conditioned medium from cells expressing LMP1(Y_{384}G), and slightly less also LMP1(A_{204}xAxA/Y_{384}G), still activated ERK. This might be due to the same effects, which were discussed in relation to Akt activation.

In summary, the presented results demonstrate that, similar to MEFs, a cocktail of selected cytokines and growth factors including LIF is induced in a CTAR2-dependent manner by native LMP1 in nasopharyngeal carcinoma cells. Furthermore, STAT3 and ERK are activated in CNE-L cells by conditioned medium of LMP1-expressing CNE-L cells. Mutation of CTAR2 abolished this indirect activation of STAT3 and reduced the phosphorylation of ERK.

4.4.10 NGFR-LMP1 Induces Cytokines and Growth Factors and Activates STAT3 through an Indirect Activation Loop in CNE-L Cells

Slight differences in signaling were obvious when comparing the expression of native LMP1 in CNE-L cells to inducible NGFR-LMP1 in MEFs. Additionally, it is difficult to discriminate direct from indirect signaling events when investigating constitutive LMP1 signaling. Therefore CNE-L cells were stably transfected with inducible NGFR-LMP1wt or NGFR-LMP1(Y_{384}G) to study inducible NGFR-LMP1 signaling in CNE-L cells. Due to administrative regulations this could not be achieved by retroviral transduction like in the MEFs. Instead, CNE-L cells were transfected with an expression vector encoding NGFR-LMP1 in addition to a hygromycin-resistance gene. After transfection the cells were cultivated in the presence of hygromycin B to select for clones with stable integration of the plasmid into the genome. The obtained single clones were tested for stable expression of NGFR-LMP1 by flow cytometry and immunoblotting (data not shown). Clone 21 presented the best expression of NGFR-LMP1wt and was chosen for the experiments (figure 4-30A). For as yet unclear reasons, it was not possible to establish a clone, which stably expressed NGFR-LMP1(Y_{384}G). None of the hygromycin B resistant clones expressed the

NGFR-LMP1(Y_{384}G) construct. Therefore experiments could only be conducted with CNE-L:NGFR-LMP1wt cells.

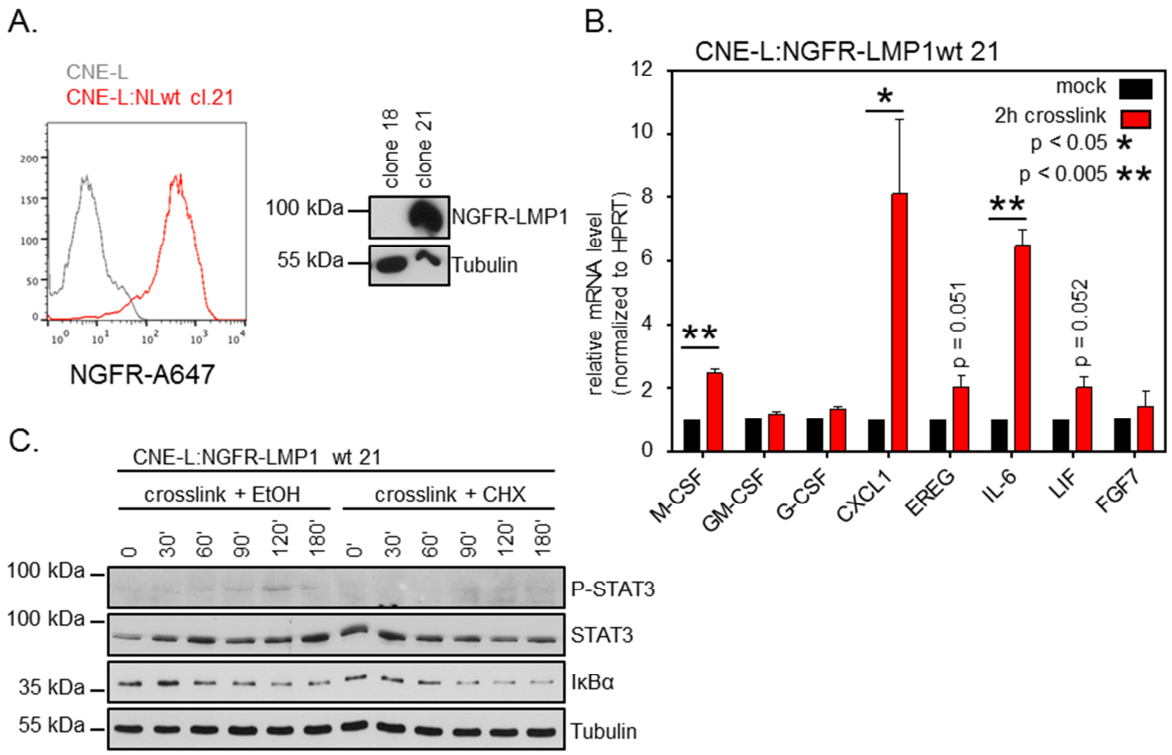

Figure 4-30. NGFR-LMP1 upregulates cytokines and growth factors, and indirectly induces STAT3 phosphorylation in the NPC cell line CNE-L. A. 5 x 10^6 CNE-L cells were transfected with 10 µg of the plasmid 1755.10 (encoding for NGFR-LMP1wt and hygromycin B phosphotransferase) and selected with 100 µg/ml hygromycin B. Clone 21 exhibited the best expression of NGFR-LMP1. FACS analysis for surface NGFR with an A647-conjugated antibody against CD119 (NGFR) demonstrated surface expression of NGFR-LMP1. Untransduced CNE-L cells were used as a control. Total expression of NGFR-LMP1 was assessed by immunoblotting for LMP1. **B.** CNE-L:NGFR-LMP1wt clone 21 cells were incubated with 1 µg/ml α-NGFR for 1 h at 37°C followed by stimulation with 10 µg/ml α-fc IgG/IgM at 37°C for 2 h, before RNA was isolated to be transcribed to cDNA. qRT-PCR was performed to investigate the expression levels of the indicated cytokines and growth factors. A non-paired heteroscedastic t-test was used to calculate the significance of the relative induction levels after 2 h stimulation compared to the non-induced control. The data were generated from 4 independent experiments. Error bars represent SD. **C.** CNE-L:NGFR-LMP1wt clone 21 cells were starved in serum-free medium overnight and stimulated by incubation with 1 µg/ml α-NGFR for 1 h at 37°C followed by 10 µg/ml α-fc IgG/IgM at 37°C for the indicated times with or without simultaneous treatment with 25 µM cycloheximide. Immunoblots for STAT3 phosphorylated at Tyr705 were performed to analyze the activation of STAT3. Degradation of IκBα served as a positive control for the stimulation and Tubulin served as a loading control.

First, the LMP1-dependent upregulation of cytokines and growth factors was assayed by qRT-PCR after 2 h of NGFR-crosslinking in comparison to an unstimulated control (figure 4-30B). To this end, the same factors were chosen for analysis that were upregulated by NGFR-LMP1wt in MEFs (chapters 4.4.4 and 4.4.5). The induction pattern resembled that of CNE-L cells transfected with native LMP1 (see chapter 4.4.9). M-CSF, CXCL1, EREG, IL-6 and LIF were induced by NGFR-LMP1wt signaling in CNE-L cells (figure 4-30B). Again, enhanced expression of GM-CSF was not detected. Notably, the overall relative induction levels in the inducible CNE-L:NGFR-LMP1wt system were much lower than the respective induction levels in the murine system, but they resembled the mRNA levels obtained from CNE-L cells transfected with native LMP1. Taken together, these results demonstrate that inducible NGFR-LMP1wt signaling leads to the upregulation of the same inflammatory cytokines and growth factors in CNE-L cells as native LMP1. Importantly, this expression pattern is reminiscent of the pattern observed after crosslinking of NGFR-LMP1wt in MEFs.

The expression of inducible NGFR-LMP1wt in CNE-L cells offered the possibility to test whether STAT3 activation in CNE-L cells by LMP1 was exclusively dependent on indirect mechanisms, as already observed in MEFs, or if LMP1 can also directly activate STAT3 phosphorylation in these cells. Therefore, CNE-L:NGFR-LMP1wt cells were tested in a crosslinking kinetics experiment in the presence or absence of cycloheximide. As expected from the relatively low induction of mRNAs, signaling activity by NGFR-LMP1 crosslinking was rather weak in CNE-L cells as compared to MEFs, which can be concluded from the incomplete degradation of IκBα after NGFR-LMP1 crosslinking (figure 4-30C). Nonetheless, 120 min crosslinking produced a faint but clear band of phosphorylated STAT3 in the immunoblot, which was not present in the cells treated with cycloheximide (figure 4-30C). This demonstrated that LMP1-induced STAT3 activation in CNE-L cells is also dependent on the de novo upregulation of soluble factors through LMP1 signaling.

4.4.11 Growth Factors and Cytokines are Upregulated in P493-6 LCLs in an LMP1-Dependent Manner

B cells are the primary target of EBV and infection with the virus transforms B cells into continuously growing lymphoblastoid cell lines (LCLs) (see introduction). After learning that LMP1 is capable of upregulating inflammatory cytokines, especially LIF, in MEF and CNE-L cells, the question arose whether this was also true for B cells. To address this question, P493-6 cells were chosen to be used for qRT-PCR experiments. P493-6 are LCLs, whose proliferation can be regulated by two separate systems. They are a subclone of the EREB 2-5 cell line, in which the activity of EBNA2 can be regulated by estrogen. Cultivation of these cells in the presence of estrogen induces the EBNA2-dependent expression of the EBV latent genes by

activation of an estrogen-receptor-EBNA2 (ER/EBNA2) fusion protein. This leads to the expression of LMP1 and, with that, an LCL phenotype (Kempkes et al, 1995). Additionally, P493-6 cells carry a tetracyclin-regulated *c-myc* allele. In the absence of tetracyclin (or its analogue doxycycline) and estrogen c-Myc is expressed and the EBNA2 program is shut off, and the cells display a Burkitt-like phenotype (Pajic et al, 2000; Schuhmacher et al, 1999). Thereby, P493-6 cells proliferate either LMP1-dependently (in the presence of estrogen and tetracyclin) or LMP1-independently (in the absence of estrogen and tetracyclin). This provides the opportunity to study gene expression within the same LCLs in the presence or absence of LMP1 signaling.

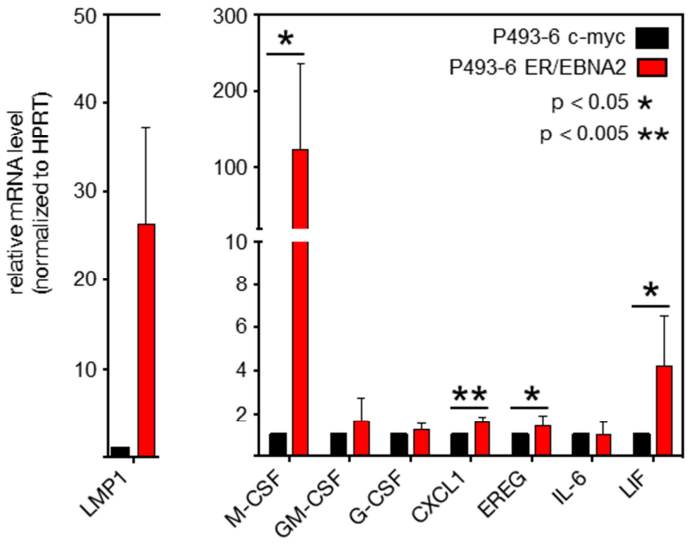

Figure 4-31. Cytokines and growth factors are induced in the LCL B cell line P493-6, when running under an ER/EBNA2 driven growth program, compared to a c-Myc driven growth program. P493-6 cells were either grown in the presence of 1 µM estrogen and 0.1 µg/ml doxycyclin to induce a growth program driven by EBNA2, or in their absence to grow in a c-Myc driven growth program. qRT-PCR was performed to assess the relative induction of LMP1 in EBNA2 driven cells. In the same way the relative induction of selected cytokines and growth factors as indicated was assayed. The significance of the induction of factors by the EBNA2 driven growth program compared with the c-Myc-program was calculated using a non-paired heteroscedastic t-test. The results were gathered from seven independent experiments.

P493-6 cells were cultivated in the presence or absence of doxycyclin and estrogen for 3 days, and total RNA was extracted to generate cDNA, which was used for qRT-PCR with primers for LMP1, M-CSF, GM-CSF, G-CSF, CXCL1, EREG, IL-6, LIF and FGF7. The results from seven separate experiments are summarized in figure 4-30.

The mRNA levels for LMP1 were highly elevated when the cells were grown under the ER/EBNA2 program compared to the c-Myc program. LMP1 expression also correlated with a significant upregulation of mRNAs for M-CSF, CXCL1, EREG and LIF. Notably, M-CSF and LIF were highly upregulated, when LMP1 was expressed in the cells. Interestingly, IL-6 levels did not vary significantly between the cells expressing c-Myc or LMP1. Levels for GM-CSF mRNA seemed to be slightly upregulated in the LMP1-expressing cells, but this difference was not statistically significant. mRNA-levels for FGF7 could not be evaluated, because FGF7 mRNA levels were too low for the detection range of the qRT-PCR.

Taken together this result demonstrates that the panel of inflammatory cytokines, which are upregulated in P493-6 LCLs in correlation with ER/EBNA2-driven LMP1 expression, is overlapping with that observed in MEFs and CNE-L cells. This again reveals that LMP1 signaling varies among different cell lines, but is still very similar and comparable in its core. Importantly, LIF is also a new target of LMP1 signaling in B cells, which might suggests a role for this cytokine in different EBV/LMP1-related diseases.

5 DISCUSSION

Although extensive research over almost 20 years has shed much light on the mechanisms of signal induction by the EBV oncoprotein LMP1, the obtained results still left some open questions and a few conflicting results. The roles of adapter proteins such as TRAFs and TRADD in LMP1 signaling have always been of particular interest. However, to date no studies existed that examined the contribution of these molecules to LMP1 signaling in a comprehensive, systematical way and within one cellular system. The present study aimed to establish such a system, in which the contributions of cellular adapter molecules to LMP1 signaling were studied with the help of TRAF6$^{-/-}$, TRAF2/5$^{-/-}$ and TRADD$^{-/-}$ MEFs. At the same time the system considered the contribution of the two CTAR domains of LMP1, and mutant LMP1 receptors with defects in CTAR1, CTAR2 or both domains were expressed in the different MEFs. In order to investigate the kinetics of signal induction, and to help discriminate direct signaling events from indirect ones, inducible NGFR-LMP1 fusion receptors were preferred over native, constitutively active LMP1. This system allowed systematical, comprehensive studies of LMP1 signaling pathways in a time dependent manner.

Different studies had shown in the past that murine systems are a powerful tool to study LMP1 signaling and its role in oncogenesis, although EBV does not naturally infect mouse cells. Rat-1 fibroblasts were first used to demonstrate the transforming potential of LMP1 (Moorthy & Thorley-Lawson, 1993; Wang et al, 1985). Mouse fibroblasts have also been established to be used as a means to study LMP1 signaling before, and LMP1 was capable of exerting its

transforming potential in these cells as well (Xin et al, 2001; Yang et al, 2000). Importantly, transgenic mice expressing LMP1 in their B cell compartment developed lymphomas with a significantly higher frequency than wildtype littermates (Kulwichit et al, 1998; Zhang et al, 2012), and expression of LMP1 in mouse epithelial cells similarly led to hyperplasia and increased papilloma formation (Shair et al, 2012; Wilson et al, 1990). Therefore murine systems and murine fibroblast cell lines represent fitting tools to study LMP1 signaling mechanisms.

In this tradition, the MEF:NGFR-LMP1 system presented in this thesis proved to be a powerful tool to study mechanisms of LMP1 signal transduction. It was possible to shed new light on the role of TRADD, and demonstrate a supporting role for this molecule in in LMP1-induced signaling. CTAR2 and TRAF6 were shown to be central regulators of various signaling pathways induced by LMP1, which involved both direct and indirect signaling mechanisms. This study is the first to demonstrate that LMP1 indirectly activates STAT3, Akt and ERK by upregulation of cytokines in a CTAR2- and TRAF6-dependent manner. Among these cytokines, leukemia inhibitory factor (LIF) was found to be a new target of CTAR2-induced signaling pathways, and to be the major inducer of indirect STAT3 activation. Furthermore, a similar, indirect activation mechanism for STAT3 was shown to be employed by LMP1 in NPC cells, and LIF was also demonstrated to be upregulated in NPC cells and LCLs. This indicates that the results obtained in the MEF:NGFR-LMP1 system can in fact be translated to other, more EBV-relevant cell lines.

Together, the data presented in this thesis, which will be discussed in the following chapters, provide new insights into signaling mechanisms employed by LMP1 and help further understanding LMP1-induced transformation and neoplasia.

5.1 Systematical, Comprehensive Analysis of LMP1-Induced Signaling Mechanisms

By using retroviral vectors, it was possible to stably express chimeric NGFR-LMP1 receptors in different MEF cell lines. Flow cytometry and immunoblotting revealed that NGFR-LMP1wt and NGFR-LMP1($Y_{384}G$) receptors were expressed in comparable amounts on the cell surface of homogenous populations of wildtype, $TRADD^{-/-}$ and $TRAF6^{-/-}$ MEFs. The cells were sorted for low expression of NGFR-LMP1, which abolished background JNK activation and IκBα degradation via spontaneous auto-aggregation of NGFR-LMP1. It was possible to induce signaling pathways by oligomerization of the receptors via antibody crosslinking. Differences occurred in the expression levels of NGFR-LMP1 depending on the integrity of the CTAR1 domain, which will be discussed in detail in chapter 5.1.4. Therefore, it was not possible to incorporate the CTAR1 mutant or the TRAF2/5-/- MEFs in the comparable, systematical analysis, and the results obtained with these MEFs cannot be directly compared to the other

receptors in a quantitative way. Induction of all investigated CTAR2-dependent signaling pathways was significantly weaker in both cell lines compared to wt MEF:NGFR-LMP1wt cells. It is likely that the lower expression levels of NGFR-LMP1 in these two cell lines account for insufficient signal induction. However, NGFR-LMP1($Y_{384}G$), in which the CTAR1 domain is functional, allowed analysis of the contribution of CTAR1 to signal transduction.

5.1.1 CTAR2 Is Essential for the Majority of Signaling Pathways Employed by LMP1

By using wildtype MEFs which stably expressed inducible wildtype or mutant NGFR-LMP1 receptors, the contribution of CTAR1 and CTAR2 to LMP1 signaling was studied in a systematical way.

First signaling events triggered by NGFR-LMP1wt were detectable as early as 20 to 30 minutes after crosslinking. Onset of signaling seems late compared to TNFα or IGF-1 stimulation, which commenced after only 5 – 10 minutes. This may be due to the fact that TNFR exists as a preformed receptor complex in the cell membrane, which allows fast signal induction after ligand binding (Chan et al, 2000). NGFR-LMP1, on the other hand, required to form patches after antibody-crosslinking, which was shown by immunofluorescence. It is unlikely that the density of the NGFR-LMP1 molecules on the cell surface plays a role in the kinetics, because the onset of inducible signaling, as shown by IκBα phosphorylation and degradation, was comparable in cells expressing low or high amounts of NGFR-LMP1.

Early induction of IκBα degradation or phosphorylation of JNK, p38 MAPK, Akt and ERK after 20 to 30 minutes of crosslinking of NGFR-LMP1wt suggests that all these pathways are directly triggered by LMP1. Notably, NGFR-LMP1 with the crippling mutation $Y_{384}G$ within CTAR2 failed to induce any of these pathways. This strongly suggests that the P_{379}VQLSY motif within CTAR2 is essentially needed to induce all of the aforementioned pathways, and that CTAR1 alone is not sufficient to induce these signaling events in MEFs. Concerning the CTAR2-dependent activation of JNK and the canonical NF-κB pathway, the results obtained in this thesis support previously described results (Boehm et al, 2010; Eliopoulos & Young, 1998; Floettmann & Rowe, 1997; Kieser et al, 1999; Kieser et al, 1997). It has also been proposed that JNK can be activated through CTAR1 dependent mechanisms in certain cell types in a TRAF1 dependent manner (Eliopoulos et al, 2003b; Kutz et al, 2008). However, NGFR-LMP1($Y_{384}G$) failed to induce JNK activation, which demonstrates that CTAR1 is not sufficient to induce this pathway in this system.

Supporting the role of CTAR2 in the activation of canonical NF-κB, luciferase reporter assays revealed a reduction of total NF-κB activity from 9.1-fold to 2.1-fold after deletion of CTAR2. Previous reports suggested that CTAR1 can induce alternative forms of NF-κB, like p65/p52

heterodimers or p50 homodimers (Song & Kang, 2010; Thornburg & Raab-Traub, 2007). The results in the present study show that CTAR1 is not involved in IκBα degradation. The CTAR2 mutant NGFR-LMP1(Y_{384}G) failed to induce degradation of IκBα. Moreover, it was possible to induce IκBα degradation by crosslinking-stimulation of NGFR-LMP1(A_{204}xAxA), even though the receptors cannot be quantitatively compared to NGFR-LMP1wt.

Similarly, NGFR-LMP1(A_{204}xAxA) was capable of activating JNK, p38 MAPK, ERK and Akt (data not shown). Although it was impossible to gain insight in the strength of those signals compared to NGFR-LMP1wt due to the aforementioned insufficient expression of the CTAR1 mutant receptor, it became clear that the P_{204}xQxT motif within the CTAR1 domain is not essentially needed for the induction of those pathways in MEFs. So far, the ERK and PI3K/Akt pathways have mostly been reported to originate at CTAR1 (Dawson et al, 2003; Lambert & Martinez, 2007; Mainou et al, 2005; Mainou et al, 2007). However, a few studies suggest that CTAR2 might also be capable of inducing these pathways (Gewurz et al, 2012; Shair et al, 2008). My results shed new light on this apparent conflict, which will be discussed in more detail in chapter 5.1.6.

Taken together, it became clear that NGFR-LMP1 critically depends on CTAR2(Y_{384}) in order to induce the canonical NF-κB pathway, MAPK pathways and PI3K/Akt signaling in MEFs, while CTAR1 is not essentially involved in the triggering of any of these pathways. As mentioned before, comparison of these results with published data leads to conflicting conclusions in some respects. Possibly, LMP1 signaling differs in various cell types, because certain components of signal transduction may not be as abundant in one cell, prompting LMP1 to induce a certain signaling pathway by other means. This would support the view that this viral oncogene is capable of adapting dynamically to cellular environments, but also that LMP1 has a range of possibilities to induce signaling pathways. This model is supported by studies using patient-derived samples of LMP1-expressing tissues or LMP1 strain variants. For instance, strength and kinetics of NF-κB and MAPK signaling pathways differ among a number of PTLD-derived LMP1 variants expressed in BL41 B cells (Vaysberg et al, 2008). Similarly, strain variants of LMP1 are selectively expressed in different tissues. While the B59.8 and Med- strains, for example, were often found in peripheral blood samples of NPC patients, they were not reported to be detected in the tumor tissue, speaking for a selection against this strain in NPC (Edwards et al, 2004). Since all LMP1 strain variants also differ slightly with respect to signaling strength (Mainou & Raab-Traub, 2006; Vaysberg et al, 2008), it seems plausible that LMP1 adapts to different cellular environments by inducing signaling pathways through different means. This knowledge may aid to understand the role this oncogene plays for different diseases in certain cell types.

5.1.2 TRAF6 is a Central Mediator of LMP1-CTAR2 Signaling

TRAF6 has been shown to be essential for CTAR2-mediated signaling and JNK, p38 MAPK and canonical NF-κB activation in several studies (Luftig et al, 2003; Schneider et al, 2008; Schultheiss et al, 2001; Wan et al, 2004). The systematical approach used in this thesis to study LMP1 signaling considered examining the role of TRAF6 by utilizing TRAF6-deficient MEFs.

No phosphorylation of p38 or JNK, and no degradation of IκBα was observed during the crosslinking-stimulation of NGFR-LMP1wt in TRAF6$^{-/-}$ MEFs. The essential role for TRAF6 in CTAR2-mediated NF-κB activation was further proven by luciferase reporter assays, where no reporter activity was detected after expression of LMP1(A_{204}xAxA) in TRAF6$^{-/-}$ MEFs. The same assay further demonstrated that TRAF6 does not play a critical role in CTAR1-mediated induction of NF-κB, because LMP1(Δ371) was capable of inducing reporter activity in TRAF6$^{-/-}$ MEFs, which likely represents non-canonical NF-κB induction via the P_{204}xQxT motif. These results support and confirm previously published results, which demonstrated a critical role for TRAF6 for the induction of these pathways (Luftig et al, 2003; Schultheiss et al, 2001; Wu et al, 2006).

Interestingly, no phosphorylation of Akt or ERK could be induced by NGFR-LMP1wt crosslinking in TRAF6$^{-/-}$ MEFs. This was also true for NGFR-LMP1(Y_{384}G), as mentioned before, and underlines the close association of TRAF6 and CTAR2 in LMP1 signaling. The importance of this new finding will be discussed in detail in chapter 5.1.6. Similarly, the critical involvement of TRAF6 in LMP1-mediated STAT3 activation will be part of chapter 5.2.

For the first time, reconstitution experiments with TRAF6 were conducted during the course of this thesis to prove that LMP1 signaling defects in TRAF6$^{-/-}$ MEFs can be rescued by expression of TRAF6. Retroviral transduction of TRAF6$^{-/-}$ MEFs:NGFR-LMP1wt cells with Flag-tagged TRAF6 ensured stable expression of TRAF6, and IRES-mediated co-expression of CFP provided the opportunity to regulate TRAF6 levels by flow cytometry sorting. However, although the obtained cells were sorted for low expression of CFP, TRAF6 was highly overexpressed in the cells compared to wildtype MEFs. This is probably due to the TLR, which is presumably much stronger than the endogenous TRAF6 promoter. Nonetheless, it was possible to induce degradation of IκBα, as well as phosphorylation of JNK and Akt after NGFR-LMP1 crosslinking in the TRAF6-rescued cells. The kinetics of signal induction were comparable to wt MEF:NGFR-LMP1wt cells, suggesting that the re-introduction of TRAF6 resulted in a re-establishment of the CTAR2-dependent signaling complex similar to wildtype MEFs. This proves not only that TRAF6 is critically important for those signaling pathways by LMP1. It also demonstrates that the

defects in signal induction by NGFR-LMP1wt in TRAF6$^{-/-}$ MEFs were a result of the lack of TRAF6 and not an off-target effect.

However, the strength of the signals fell short of the wildtype MEF control. It was neither possible to detect any substantial phosphorylation of ERK or STAT3 after NGFR-LMP1wt crosslinking in TRAF6-rescued MEFs. One possible explanation for this might be the massive exogenous overexpression of TRAF6 after viral transduction, which disrupts the natural stoichiometry of signaling proteins in the cell.

In the future, the TRAF6 rescue system will have to be optimized with regard to TRAF6 expression levels. This may be achieved by using a different and weaker promoter for TRAF6 expression to reduce the expression levels of the protein after rescue. This system will then be used to incorporate different TRAF6 mutants into the study to evaluate the precise molecular mechanisms, by which TRAF6 is utilized to mediate LMP1 signaling. This includes mutants which are not able to bind to LMP1, as proposed by Fabian Giehler (Giehler, 2012), but also mutants lacking functional E3 ligase activity, which apparently has differential effects on JNK, p38 and NF-κB signaling (Schultheiss et al, 2001).

5.1.3 The Role of TRADD in LMP1-Induced Signaling

By using TRADD knockout MEFs, it became evident that the death domain protein TRADD plays a supporting role in CTAR2-dependent LMP1 signaling in murine fibroblasts. Crosslinking-stimulation of NGFR-LMP1wt in TRADD$^{-/-}$ MEFs resulted in induction of IκBα degradation. However, the onset of IκBα degradation was marginally shifted from 30 to 45 minutes in TRADD$^{-/-}$ MEFs, which suggests that TRADD contributes to the activation of the canonical NF-κB pathway, but is not essentially needed for its induction. This result was supported by NF-κB luciferase assays. The lack of TRADD reduced the induction of total NF-κB activity by more than 50% compared to wildtype MEFs, but did not fully abolish the signal. Accordingly, the CTAR1 mutant LMP1(A_{204}xAxA), which is only capable of inducing canonical NF-κB signals via the CTAR2 domain, activated NF-κB to the same level as LMP1wt in TRADD$^{-/-}$ MEFs, which further supports a non-essential role for TRADD in MEFs. These results seemingly contradict published results, which showed that IKK2 kinase activity, which is a hallmark of the canonical NF-κB pathway, was not induced in TRADD$^{-/-}$ B cells upon LMP1 expression (Schneider et al, 2008). In the B cells described by Schneider et al. TRADD was absolutely required to recruit IKK2 to CTAR2 (Schneider et al, 2008). Therefore it is possible that MEFs and B cells differ with regard to the importance of TRADD-dependent IKK2 recruitment in order to activate the canonical NF-κB pathway. TRADD might be differentially essential to stabilize the CTAR2 complex in different cell lines.

It is possible that TRAF6, which is a key player in signal transduction at CTAR2, could induce a signaling complex that does not critically rely on TRADD and is more reminiscent of Toll/IL-1 receptors than the TNFR family (see chapter 1.5.1). TRADD has not been implicated in TIR-like signaling to induce canonical NF-κB, with the exception of TRIF-dependent TLR3 and TLR4 (Pobezinskaya et al, 2008). Furthermore, IRAK-1, which is a component of the TIR-like signaling complex, is also involved in NF-κB signaling by LMP1 (Luftig et al, 2003; Song et al, 2006). Other facts also argue against a critical involvement of TRADD in CTAR2-dependent signaling and the recruitment of the signaling complex. TRADD had been proposed to directly bind to LMP1-CTAR2 to induce the formation of a signaling complex (Izumi et al, 1999b; Izumi & Kieff, 1997). However, TRAF6 is more likely to play this role. TRAF6 was shown to directly bind TNIK to the LMP1 signaling complex, which recruits the TAB/TAK complex to activate IKKs (Shkoda et al, 2012). TRAF6 itself was recently shown to directly bind to LMP1-CTAR2 (Giehler, 2012). This links the TAB/TAK complex to LMP1 via TRAF6 and TNIK, suggesting that TRADD does not play and essential role in the formation of the canonical NF-κB signalosome upstream of TAK/TAB, as it was previously suggested by the direct association of TRADD with LMP1 (Izumi & Kieff, 1997). Another possibility is that LMP1 does not solely depend on the activity of IKK2 to induce canonical NF-κB in MEFs. It is known that IKK1, in concert with NEMO, is sufficient to induce this pathway after IL-1 stimulation (Solt et al, 2007). Furthermore it was shown that LMP1 is capable of inducing IκBα degradation in IKK2 knockout cells, albeit not to the full extent (Gewurz et al, 2012). Similarly, NGFR-LMP1wt potently induced IκBα degradation in IKK2$^{-/-}$ MEFs, hinting at an NF-κB inducing signaling mechanism bypassing IKK2 in murine fibroblasts (data not shown). Therefore TRADD may be more important for LMP1 signaling in certain cell types than in others. The recruitment of IKK2 to the signaling complex may be much more dependent on TRADD in B cells than in fibroblasts, or the components of the NF-κB inducing IKK complex might be more restricted in B cells.

The lack of TRADD markedly reduced NGFR-LMP1wt-induced activation of MAPK pathways. This suggests that activation of JNK, ERK and p38 MAPK is much more sensitive than the canonical NF-κB pathway regarding the supporting role of TRADD in LMP1 signaling. It was shown before that dominant negative TRADD lacking the death domain is capable of blocking p38 MAPK activity (Schultheiss et al, 2001). Results shown in this thesis demonstrate that NGFR-LMP1 is able to induce p38 phosphorylation, implicating that TRADD is not critically needed for this pathway, but plays an important enhancing role in certain cell types. Studies using constitutively active native LMP1 have so far reported that TRADD is not involved in JNK signaling by LMP1 (Kieser et al, 1999; Schneider et al, 2008; Wan et al, 2004). It is possible that in the case of constitutively active LMP1, the contributing role of TRADD is less prominent.

Positive as well as negative feedback mechanisms might lead to comparable JNK activation during steady-state signaling in cells with or without TRADD. Therefore the supporting role of TRADD in JNK activation became more obvious in the transiently inducible system used in this thesis. This example demonstrates how the inducible NGFR-LMP1 system can contribute to understanding details and nuances of LMP1-induced signaling.

Strikingly, IκBα protein was not sufficiently re-expressed after NGFR-LMP1-induced degradation in TRADD$^{-/-}$ MEFs. While total IκBα started to reappear after 180 minutes crosslinking-stimulation of NGFR-LMP1wt in wildtype MEFs, this did not occur in TRADD$^{-/-}$ MEFs. It is possible that TRADD influences signaling events downstream of IκBα, which cause IκBα re-synthesis via canonical NF-κB-induced gene transcription (Gewurz et al, 2012). Since MAPK signaling by LMP1 was also diminished in TRADD$^{-/-}$ MEFs, it is possible that these pathways also contribute to the re-introduction of IκBα. Future experiments will have to focus on the role of TRADD in canonical NF-κB activation in more detail, and the nuclear translocation and activity of NF-κB dimers or their DNA-binding abilities in TRADD$^{-/-}$ cells should be evaluated.

In summary, the results shown in this thesis suggest that TRADD plays an important supportive role in the signal induction at CTAR2 of LMP1 in MEFs. The reduced activation of MAPK and NF-κB pathways by NGFR-LMP1wt in TRADD$^{-/-}$ MEFs coincides with the abolished activation of these pathways in TRAF6$^{-/-}$ MEFs and by NGFR-LMP1(Y_{384}G). This suggests that both TRAF6 and TRADD are present in the same signaling complex that is recruited at CTAR2 via Y_{384}. In fact, TRAF6 directly binds to CTAR2, and a direct interaction of TRAF6 and TRADD was recently shown (Giehler, 2012). Fabian Giehler suggested in his thesis that TRADD might play a stabilizing role within the signaling complex at CTAR2 and might be helping to achieve higher order multimeric complexes consisting of multiple LMP1 oligomers in association with several TRAF6 trimers (Giehler, 2012). The reduced response to NGFR-LMP1-induced signaling in TRADD$^{-/-}$ MEFs functionally extends this model. Lack of TRADD might lead to a less stable CTAR2 signaling complex, or to reduced recruitment of TRAF6. This in turn would result in a diminished activation of CTAR2-dependent signaling pathways by TRAF6.

This in mind, it will be interesting to study the role of TRADD for LMP1 signaling in more detail. Directly comparing different cell types, especially cell types directly associated with EBV-mediated diseases such as PTLD, NPC or HL, with regard to the importance of TRADD during transformation, might give valuable hints towards focused treatment of these diseases. Knowing that the impact of TRADD on LMP1 signaling varies depending on the cell type or possibly even the state of transformation might aid specialized drug development targeting LMP1 signaling.

5.1.4 Constitutive Activation of the Non-Canonical NF-κB Pathway by NGFR-LMP1 – Hints Towards Tonic LMP1 Signaling?

LMP1 has long been known to engage the non-canonical NF-κB pathway, and it was shown that the CTAR1-domain, and more precisely the TRAF-binding site therein, was responsible for this pathway (Atkinson et al, 2003; Luftig et al, 2004; Saito et al, 2003). Moreover, studies conducted with dominant negative TRAF2 and TRAF3 suggested that both molecules play inhibiting roles in the processing of p100 to p52 by LMP1, although TRAF3 was not reported to be degraded in the process (Brown et al, 2001; Song & Kang, 2010). In the course of this study the examination of the non-canonical NF-κB pathway was part of the systematical, comprehensive analysis of LMP1 signaling. The obtained results demonstrated that p100 to p52 processing was strictly depending on the integrity of the P_{204}xQxT site within CTAR1, as published before, and neither mutation of Y_{384} within CTAR2 nor the lack of TRAF6 or TRADD had any effect on the processing of p100 to p52.

However, induction of p100 processing by NGFR-LMP1 was independent of crosslinking, and high levels of the active degradation product p52 were found in MEFs regardless of stimulation, while p100 levels were low. Crosslinking did not detectably further decrease p100 protein levels. Nonetheless, a slight increase of p52 could be detected after 180 minutes of crosslinking, which suggests that the pathway activation was enhanced by crosslinking. However, this rise in p52 levels was only present in wildtype and $TRADD^{-/-}$ MEFs expressing NGFR-LMP1wt, and it coincided with a simultaneous increase in p100 levels. Both p100 and p52 were not increased in $TRAF6^{-/-}$ MEFs or cells expressing the CTAR2 mutant NGFR-LMP1(Y_{384}G), demonstrating that p100 was upregulated via CTAR2-dependent signaling. Activation of canonical NF-κB is likely responsible for this p100 induction, as previously demonstrated (Atkinson et al, 2003). Importantly, activation of p100 degradation depended on CTAR1. The mutation of the TRAF binding site to A_{204}xAxA completely abolished the activation of this pathway, which is consistent with published data (Atkinson et al, 2003; Luftig et al, 2004; Saito et al, 2003). Interestingly, the constitutive activation of p100 processing in the absence of crosslinking is a clearly LMP1-CTAR1-dependent effect. No other LMP1-induced pathways were constitutively active in the absence of crosslinking after the cells had been sorted for low expression of the receptor, which suggests that spontaneous auto-aggregation of the receptor was very low or even absent. Immunofluorescence also showed that NGFR-LMP1 aggregated only in the presence of antibody-crosslinking. Furthermore, no NGFR-LMP1 was found in lipid rafts prior to crosslinking.

It must therefore be considered that the activation of the non-canonical NF-κB pathway by LMP1 does not require pronounced, forced aggregation of the receptor. Instead, the activation mechanisms must be much more sensitive compared to other pathways. Possibly lower-order

aggregation of the receptors is enough to trigger the non-canonical NF-κB pathway, or even the monomeric molecule might suffice. Monomeric LMP1 is thought to be incapable of inducing any signaling. Studies with truncated versions of LMP1, which lacked the first four transmembrane domains necessary for oligomerization, were shown to be inefficient to induce NF-κB-dependent gene transcription (Gires et al, 1997; Mitchell & Sugden, 1995). However, close re-examination of the published data reveals that expression of the truncated “one-finger” LMP1 mutant did in fact induce residual levels of NF-κB activity, even if full-length LMP1 was significantly more efficient (Gires et al, 1997; Mitchell & Sugden, 1995). The “one-finger” mutant still induced NF-κB 2- to 3-fold in comparison to the empty vector control (Mitchell & Sugden, 1995), which suggests that monomeric LMP1 might in fact be capable of inducing residual signaling events. At the time, this was considered to be irrelevant. However, the data presented in this thesis suggest that the observed residual activation might in fact be relevant. The study by Gires et al. also demonstrated that forced crosslinking of “one-finger” LMP1 induced NF-κB reporter activity, and that this activity could be further increased by transfection of increasing amounts of the receptor (Gires et al, 1997). At the same time, increasing amounts of unstimulated “one-finger” LMP1 also resulted in increasing levels of NF-κB induction (Gires et al, 1997). This reflects the fact that an overabundance of LMP1 on the cell surface might lead to spontaneous triggering of signaling pathways due to random auto-aggregation, and that activation of NF-κB seems to be highly sensitive to these spontaneous events. The data obtained in the course of this thesis demonstrate that the degradation of IκBα as a hallmark of the canonical NF-κB pathway relies heavily on the induced crosslinking of NGFR-LMP1. At the same time, constitutive processing of p100 to p52 could not be diminished even by reducing the amounts of NGFR-LMP1 on the cell surface. Therefore, it must be considered that the induction of the non-canonical NF-κB pathway is much more sensitive compared to the canonical pathway. Furthermore, no NGFR-LMP1 was present in lipid rafts fractions in the absence of crosslinking, suggesting that the constitutive activation of the non-canonical NF-κB pathway does not require LMP1 to localize to this membrane compartment.

One possible explanation for this constitutive signaling of monomeric NGFR-LMP1 might be that LMP1 is capable of inducing “tonic” signaling events that are independent of aggregation, or that are sensitive to only very low levels of oligomerization. Of course, naturally occurring LMP1 does not require a ligand for oligomerization and constitutively aggregates via its transmembrane domains. Therefore “tonic” signaling might not play a role for native wildtype LMP1. However, a truncated 28 kDa form of LMP1 occurs naturally and encompasses the C-terminal two-thirds of LMP1 corresponding to transmembrane domains 5 and 6 as well as the signaling domain (Hudson et al, 1985). The protein, which was later termed lytic LMP1, is expressed late during

virus replication in the lytic phase (Hudson et al, 1985; Modrow & Wolf, 1986; Wang et al, 1988). To date, the functional role of lytic LMP1 remains largely unknown. It was shown that co-expression of lytic LMP1 with full-length LMP1 inhibits NF-κB activation by full-length LMP1 in a dose-dependent manner, but at the same time lytic LMP1 exerts some minor activation of NF-κB itself (Erickson & Martin, 2000). Functionally, lytic LMP1 may play a critical role in the production of progeny virus and was associated with the budding of newly forming virions (Ahsan et al, 2005; Vazirabadi et al, 2003). Interestingly, full-length LMP1 can functionally replace lytic LMP1 in its role in virus replication (Ahsan et al, 2005), suggesting that both molecules share similar properties concerning their molecular and functional impact on the cell and on lytic replication. Possibly, signaling similar to the “tonic” LMP1 signaling described herein plays a role in lytic virus replication. The high sensitivity of non-canonical NF-κB induction by unstimulated NGFR-LMP1 presented in this thesis supports the fact that lytic LMP1 might in fact be capable of actively inducing signal transduction, just as the residual activation of NF-κB by truncated or lytic LMP1 in previous studies suggests (Erickson & Martin, 2000; Gires et al, 1997; Mitchell & Sugden, 1995). It would be intriguing to learn if the molecular function of lytic LMP1 depends on induction of the non-canonical NF-κB pathway, as suggested by the results presented in this thesis.

If (lytic) LMP1 is a receptor capable of inducing tonic signaling events, this might also raise the possibility that other receptors of the TNFR-family, which LMP1 is closely related to in terms of signaling mechanisms, could be capable of or even dependent on tonic signaling as well. CD40 is the TNFR-family member most comparable to LMP1 (Kaykas et al, 2001; Kilger et al, 1998; Rastelli et al, 2008; Uchida et al, 1999). As a matter of fact, primary splenic B cells also constitutively process low levels of p100 to p52, and RelB as well as p52 can be found in the nuclear fraction of these cells, all of which can be increased by inducing CD40 signaling (Hömig-Hölzel et al, 2008). It would be interesting to learn in the future if this residual activity of the non-canonical NF-κB pathway in B cells originates at the CD40 receptor, and if mechanisms similar to the described, putative tonic LMP1 signaling lead to this.

As mentioned before, NGFR-LMP1 receptor expression was insufficient when CTAR1 was mutated or if the cells lacked TRAF2/5. Flow cytometry revealed that both cell lines never homogenously expressed the chimeric receptor, as it was found in wildtype MEFs expressing NGFR-LMP1wt, for example. Instead, two distinct cell populations were present, with one expressing the expected high amounts of NGFR-LMP1 on the cell surface, and a second population expressing significantly lower amounts of the receptor, although all cells had been equally sorted beforehand. Accordingly, total NGFR-LMP1 protein levels were considerably lower in wt MEF:NGFR-LMP1(A_{204}xAxA) cells than wt MEF:NGFR-LMP1wt, which could be seen

in immunoblots. This suggests three possible explanations: (1) Cells within this lower expression population must have been under selective pressure to down-regulate surface expressed NGFR-LMP1, or lower amounts of NGFR-LMP1 poses a survival advantage in the absence of CTAR1. (2) Cells with lower amounts of NGFR-LMP1, which is defective in CTAR1 signaling, have a proliferative advantage, which enables them to grow faster and over-grow the other cells. (3) NGFR-LMP1, which is incapable of CTAR1-dependent signaling, cannot be sufficiently expressed on the cell surface.

The third option seems unlikely, because this would have resulted in a more intermediate population. A distinct population exists, which expresses NGFR-LMP1 at high levels comparable to wt MEF:NGFR-LMP1wt cells. If the CTAR1 mutation caused the receptor to be insufficiently expressed, this should be true for all cells. This would have caused a more or less even distribution of cells with lower amounts of NGFR-LMP1 in the FACS profile.

Noteworthy, both the CTAR1 P_{204}xQxT-motif and TRAF2 were shown to be involved in the induction of the non-canonical NF-κB pathway (Atkinson et al, 2003; Luftig et al, 2004; Song & Kang, 2010), and this pathway is constitutively active in MEFs expressing NGFR-LMP1 even in the absence of a stimulus. This leads to the assumption that the lack of “tonic” LMP1-CTAR1 signaling might somehow be connected to the flawed surface expression of NGFR-LMP1 when CTAR1 is mutated or TRAF2/5 are lacking. It is possible that CTAR2 as well exhibits some minor constitutive activity, which stays below the detection sensitivity of the immunoblot assays used in this study. In fact, basal amounts of phosphorylated IκBα were found in unstimulated wt MEF:NGFR-LMP1wt before they were sorted for low amounts of GFP. Different groups have shown that high levels of LMP1 have cytostatic or even cytotoxic effects on cells (Hammerschmidt et al, 1989; Kaykas & Sugden, 2000; Le Clorennec et al, 2008). Spontaneous apoptosis of LCLs was shown to be dependent on overexpression of LMP1, which led to NF-κB induced Fas upregulation and activation of caspases (Le Clorennec et al, 2008; Le Clorennec et al, 2006). Therefore one can speculate that the CTAR1 domain somehow counter regulates any detrimental effects of basal NGFR-LMP1 CTAR2 signaling in MEFs. Minimal “tonic” induction of canonical NF-κB by CTAR2 may be sufficient to mediate some sort of pressure on the cells, which is balanced by “tonic” signaling through CTAR1. This assumption can be backed by the fact that mutation of both CTAR1 and CTAR2 did not lead to such drastic effects concerning surface expression of NGFR-LMP1, and wt MEF:NGFR-LMP1(A_{204}xAxA/Y_{384}G) were not distributed in two separate populations in the flow cytometry assay. Furthermore, expression of lytic LMP1 inhibits NF-κB activation by full-length LMP1, as mentioned before (Erickson & Martin, 2000). It is possible that tonic signaling via CTAR1 of lytic LMP1 is responsible for this

effect, suggesting a mechanism by which CTAR1 signaling is required to down-regulate CTAR2-dependent NF-κB activation, which might be harmful for the cells.

Nonetheless, as of now it remains unclear why two distinct populations of wt MEF:NGFR-LMP1(A_{204}xAxA) and TRAF2/5$^{-/-}$ MEF:NGFR-LMP1wt cells formed, instead of an intermediate, heterogenous population.

5.1.5 The Role of TRAF3 in the Induction of the Non-Canonical NF-κB Pathway by LMP1

TRAF3 is an important negative regulator of the non-canonical NF-κB pathway by sequestering NIK to a complex with TRAF2 and cIAPs, which mediates its continuous degradation (see introduction). It has been shown that TRAF3 binds to the P_{204}xQxT motif within CTAR1 of LMP1 (Devergne et al, 1996), and overexpression of TRAF3 could reduce LMP1-induced degradation of p100 to p52 (Song & Kang, 2010). Together this suggests that TRAF3 is also involved in the regulation of non-canonical NF-κB signaling by LMP1. However, studies in B cells showed that, in contrast to CD40 signaling, no degradation of TRAF3 is induced through LMP1-dependent mechanisms (Brown et al, 2001). To study the exact contribution of TRAF3 to LMP1 signaling, TRAF3$^{-/-}$ MEFs would have been required, but these cells had not been available.

The systematical analysis of LMP1-induced signaling mechanisms described in this thesis revealed that cytosolic TRAF3 levels are indeed reduced upon NGFR-LMP1 crosslinking. This effect was independent of TRAF6 and TRADD, which are cellular mediators of other LMP1 signaling pathways like the canonical NF-κB pathway or MAPK pathways. However, reduction of TRAF3 levels upon NGFR-crosslinking strictly depended on the CTAR1 TRAF-binding site and no TRAF3 reduction was observed when the P_{204}xQxT motif was mutated to A_{204}xAxA. Importantly, this reduction in protein levels did not reflect degradation of TRAF3, as treatment of the cells with the proteasome inhibitor MG132 did not prevent TRAF3 reduction. Instead, TRAF3 was redistributed to an NP-40 insoluble fraction. These results support the data obtained by Brown et al. (Brown et al, 2001), and suggest that TRAF3 is relocated into distinct cellular compartments upon LMP1-induced signaling instead of being degraded.

A similar mechanism of TRAF3 redistribution has already been reported for two other receptors. The cellular BAFF receptor and the viral oncoprotein Tio of *Herpesvirus ateles* both induce TRAF3 redistribution instead of proteasome-dependent degradation to induce the non-canonical NF-κB pathway (de Jong et al, 2013; Varfolomeev et al, 2012). The redistribution of TRAF3 leads to a reduction of the protein in the cytosol, which culminates in the liberation and subsequent stabilization of NIK, similar to the degradation of TRAF3. On the basis of the data obtained in this thesis, it is plausible to suggest that LMP1 utilizes similar mechanisms of TRAF3 redistribution to induce signaling.

It is possible that the increase in p52 levels after crosslinking-stimulation of NGFR-LMP1wt in wildtype and TRADD$^{-/-}$ MEFs is a result of this induced TRAF3 relocation. However, it is unclear how the constitutive CTAR1-dependent processing of p100 in the absence of crosslinking can take place, if TRAF3 is present in the cells. Even more so, basal TRAF3 levels were markedly increased in MEFs expressing NGFR-LMP1wt compared to untransduced MEFs. Increased TRAF3 levels should inhibit the activation of the non-canonical NF-κB pathway by sequestration of NIK, as it was shown by TRAF3 overexpression (Song & Kang, 2010). Therefore, TRAF3 itself is not only upregulated by tonic LMP1 signaling, but the constitutive processing of p100 is induced without depletion of TRAF3. This suggestion is even further substantiated by the fact that NGFR-LMP1 is only found in lipid rafts after crosslinking-stimulation. TRAF3 was found to be associated with LMP1 only within lipid rafts and never outside (Briseno-Franke, 2006), strongly suggesting that tonic activation of non-canonical NF-κB by NGFR-LMP1 is triggered outside of lipid rafts and does not require TRAF3 recruitment. These findings further suggest that liberation and stabilization of NIK is possibly not needed for the constitutive processing of p100 by unstimulated NGFR-LMP1.

The events leading to p52 release downstream of NIK stabilization include IKK1 activation and the subsequent phosphorylation and processing of p100. In the aforementioned scenario LMP1-CTAR1 would have to be capable of inducing either the activation of IKK1 or the processing of p100 directly. The exact activation mechanisms of this NIK-independent LMP1-induced non-canonical NF-κB signaling must be investigated in more detail in the future. Even if this mechanism does not play a critical role for full-length LMP1 signaling, which seems to involve TRAF3 recruitment, it could help understanding the function and functionality of lytic LMP1, but might additionally reveal another, minor signaling mechanism by LMP1, which might aid LMP1-induced transformation of cells.

To suggest one possibility, Akt, apart from NIK, has been described as a kinase for IKK1. Akt phosphorylates and thereby activates IKK1 at Thr23 in response to IL-1 and TNFα (Cahill & Rogers, 2008; Ozes et al, 1999). It was shown that inhibition of Akt activity by treatment of MEF cells with the PI3K-inhibitor LY294002 reduces the basal processing of p100 in these cells (Gustin et al, 2006). Furthermore, basal p52 levels were lower in Akt$^{-/-}$ MEFs than in wildtype MEFs, and stimulation of LTβR (lymphotoxin β receptor) failed to induce p100 to p52 processing in MEFs lacking Akt (Gustin et al, 2006).

LMP1 was demonstrated to activate the PI3K/Akt pathway via the CTAR1-domain, although the exact mechanisms for this remain elusive (Dawson et al, 2003; Lambert & Martinez, 2007; Mainou et al, 2005). Even though Akt phosphorylation was clearly enhanced by crosslinking of NGFR-LMP1, it is possible that tonic NGFR-LMP1-CTAR1 signaling is sufficient to induce low

levels of Akt-activity, which appear as background on the immunoblots after long exposure (data not shown). This in turn could enhance basal processing of p100, resulting in the accumulation of high levels of p52 independently of TRAF3-depletion. Akt-involvement in the constitutive processing of p100 by NGFR-LMP1 could be investigated by using an Akt inhibitor to prevent Akt activity.

Taken together, the obtained results demonstrate that LMP1 is capable of inducing the non-canonical NF-κB pathway through tonic signaling events, which in turn suggests that the truncated, lytic LMP1 might be able to induce signaling in a similar manner. It is possible that tonic signaling events are important for the maintenance of cells expressing high levels of NGFR-LMP1. Furthermore the data indicate that basal, CTAR1-induced processing of p100 is not relying on TRAF3 depletion. At last, LMP1 employs a mechanism independent of proteasomal degradation to reduce TRAF3 levels in the cytosol. Similar to the cellular BAFF receptor and the viral oncoprotein Tio, LMP1 induces the redistribution of TRAF3 into NP-40 insoluble cellular compartments.

These data can provide a basis for further studies on the potential molecular mechanisms employed by lytic LMP1 and the role it plays in viral replication. Additionally, the data further support the idea of the existence of large, interconnected signaling networks with numerous levels of regulation and balancing, instead of linear, individual signaling pathways.

5.1.6 CTAR2 Is Involved in the Early, Direct Activation of Akt and ERK

LMP1 deregulates multiple cellular signaling pathways to ensure continuous growth of the EBV host cells, and both the PI3K/Akt and the ERK pathway have been shown to be important for the transformation of LMP1-transfected cells (Dawson et al, 2003; Mainou et al, 2005; Mainou et al, 2007).

NGFR-LMP1wt induced activation of both Akt and ERK1/2 in MEFs starting at around 45 and 20 minutes of crosslinking, respectively. Phosphorylation of both proteins increased gradually after that until it peaked at around 180 minutes. This late activation was especially pronounced in the PI3K/Akt pathway, and strong phosphorylation of Akt occurred only at this late time point.

Moreover, results obtained from experiments with cycloheximide revealed a biphasic induction of both the PI3K/Akt pathway and the ERK pathway. While a mild activation of both pathways was observed at early time points during the stimulation, treatment of the cells with cycloheximide blocked the late, enhanced activation, which was especially drastic with regard to Akt phosphorylation. Pronounced Akt phosphorylation after 180 minutes of crosslinking was completely lacking in cells treated with cycloheximide. These results demonstrate that LMP1-induced protein-synthesis was essential for the strong activation of both the ERK and PI3K/Akt

pathway, which in turn demonstrates that LMP1 engages two different mechanisms to induce the two signaling pathways. The indirect activation of both pathways by CTAR2-dependent upregulation of cytokines will be discussed in chapter 5.2. The early, direct activation of both pathways is likely induced by the CTAR1 domain, as described in the literature (Dawson et al, 2003; Lambert & Martinez, 2007; Mainou et al, 2005; Mainou et al, 2007). Strikingly, activation of both pathways were completely blocked in TRAF6$^{-/-}$ MEFs and in cells expressing the CTAR2 mutant NGFR-LMP1($Y_{384}G$). If CTAR1 alone was responsible for direct activation of Akt and ERK, a weak, early activation of both pathways would have been expected after crosslinking-stimulation of either TRAF6$^{-/-}$ MEF:NGFR-LMP1wt or wt MEF:NGFR-LMP1($Y_{384}G$). However, this was not the case as no signal at all was observed, suggesting that CTAR2 contributes to direct, CTAR1-dependent Akt and ERK activation. Recent data indicate that this correlation of CTAR1 to both pathways might in fact be not as strict. Expression of the CTAR2-domain alone enhanced Akt phosphorylation in EBV-positive C666.1 cells (Shair et al, 2008), and ERK was activated in 293 cells by transfection of LMP1-CTAR2 (Gewurz et al, 2012). In fact, close examination of other published data also reveals that a strict association of the CTAR1 domain and the activation of the PI3K/Akt pathway is not thoroughly supported. For example, expression of truncated forms of LMP1 either encompassing the CTAR1 or the CTAR2 domain (LMP1(1-231) corresponding to the CTAR1 domain, or LMP1(del187-352) corresponding to the CTAR2 domain) in HEL fibroblasts caused similar levels of phosphorylated Akt (Mainou et al, 2005). Possibly, direct activation of Akt and ERK signaling, which is likely CTAR1-dependent, also requires the functionality of the CTAR2 signaling complex in MEFs in the context of inducible NGFR-LMP1. It was proposed before that CTAR1 and CTAR2 can cooperate to induce signaling (Busch & Bishop, 2001). It is possible that pronounced direct activation of the PI3K/Akt and ERK pathways relies on the presence of both signaling regions in MEFs. Another possibility is that LMP1 relies on the recruitment of TRAF6 to the signaling complex to properly activate PI3K/Akt, which is facilitated by CTAR2. Several reports have demonstrated a role for TRAF6 in Akt activation. TRAF6-dependent ubiquitination of Akt was found to aid Akt recruitment to the membrane. Furthermore, TRAF6 and c-Src can form a complex that positively regulates Akt activity (Funakoshi-Tago et al, 2003; Wong et al, 1999). In fact, LMP1 activates Src kinase family members, and chemical inhibition of the tyrosine kinase c-Src or its downstream target Syk blocked LMP1-induced Akt phosphorylation (Hatton et al, 2012). Based on these data and the data presented in this thesis, I suggest a model by which LMP1 activates the PI3K/Akt pathway by recruiting TRAF6 via its CTAR2 domain to form a complex with c-Src. This complex is needed to facilitate activation of Akt, possibly in concert with CTAR1-mediated PI3K recruitment. However, mutation of CTAR1 did not abrogate Akt phosphorylation after NGFR-LMP1-crosslinking (data not shown). Therefore, CTAR1-dependent activation of PI3K may not

be a mandatory step for LMP1-induced PI3K/Akt signaling. Similarly, ERK was reported to be activated by Src kinase family members specifically via Raf-1 (Fabian et al, 1993), although this pathway has, to my knowledge, not been shown for LMP1-induced signaling so far. However, CD40-dependent activation of ERK was shown to rely on TRAF6, and TRAF6-mediated ERK activation could be quenched by dominant negative Raf (Kashiwada et al, 1998). It is intriguing that LMP1 might be able to directly activate ERK via a similar TRAF6-Src-dependent mechanism as Akt.

Future experiments will have to further evaluate if the LMP1-mediated CTAR2-TRAF6-Src-Akt/ERK axis exists to evaluate and understand the importance of this particular signaling mechanism.

5.2 Indirect Activation of STAT3, Akt and ERK by LMP1

LMP1 was found to be capable of inducing the ERK and PI3K/Akt pathways through indirect means. A third pathway was shown herein to be additionally activated by this mechanism: Pronounced STAT3 phosphorylation occurred at relatively late time points during the stimulation at 90 and 120 minutes of crosslinking, and cycloheximide-treatment completely abolished this signal. This result demonstrates that activation of STAT3 is exclusively indirect and depending on *de novo* protein synthesis.

Furthermore, treatment of MEFs with medium conditioned by NGFR-LMP1wt signaling events provoked strong activation of STAT3 and slightly milder activation of Akt and ERK. This proves that all three pathways are activated by indirect signaling loops involving soluble factors. Production of these soluble factors was critically relying on signaling events originating at the CTAR2 domain, since no activation of signaling was achieved with conditioned medium harvested from wt MEF:NGFR-LMP1($Y_{384}G$) or $TRAF6^{-/-}$ MEF:NGFR-LMP1wt.

Activation of the canonical NF-κB pathway, the JNK pathway and the p38/MAPK pathway all critically rely on CTAR2 and are mediated by TRAF6. Consequently, it was of no surprise to see that inhibition of those three pathways by chemical inhibitors also led to a reduction in the activation of STAT3, Akt and ERK. More precisely, activation of STAT3 was most potently reduced by the JNK-inhibitor SP600125 and the NF-κB inhibitor JSH-23, while the p38 inhibitor SB203580 had a slightly milder effect. Akt and ERK phosphorylation were both strongly inhibited by the JNK-inhibitor, while inhibition of NF-κB had only a marginal effect on those two pathways. Notably, however, JSH-23 specifically and potently reduced the strong activation of Akt at 180 minutes. Inhibition of p38 potently blocked ERK activation but was only mildly effective on the activation of Akt. Taken together, these results strongly support the involvement of all three CTAR2-dependent pathways in the autocrine/paracrine activation of secondary signaling

pathways. STAT3 activation was also found to be diminished after crosslinking of NGFR-LMP1wt in TRADD$^{-/-}$ MEFs. CTAR2 dependent MAPK signaling was similarly reduced in TRADD$^{-/-}$ MEFs. Therefore the indirect activation of STAT3 was reduced in TRADD$^{-/-}$ MEFs likely due to the involvement of TRADD in the activation of JNK and p38 MAPK.

This finding broadens our understanding of LMP1-induced signaling. It is clear that signaling pathways do not only affect cells in a stringent, linear way, but that their outcome must be viewed on a much larger scale. It has been shown that especially the JNK and canonical NF-κB pathways are essential for LMP1-induced cellular transformation and continuous growth and proliferation (Cahir McFarland et al, 1999; He et al, 2000; Kutz et al, 2008). At the same time, activation of Akt and ERK was similarly associated with cell transformation (Dawson et al, 2003; Mainou et al, 2005; Mainou et al, 2007). The possibility for LMP1 to induce both ERK and Akt pathways through indirect mechanisms might enhance the transforming abilities of CTAR2-regulated MAPK and NF-κB pathways. Additionally, paracrine effects can affect neighboring cells and lead to enhanced tumor growth.

Activation of STAT3 by LMP1 critically relied on the upregulation of soluble factors through CTAR2- and TRAF6-dependent activation of canonical NF-κB, JNK and to a slightly lesser extent p38. This is a novel finding and extends our understanding of STAT3 activation by LMP1. STAT3 activation had been implicated to be induced by autocrine signaling before, and a neutralizing antibody against IL-6 reduced STAT3 phosphorylation in LMP1 expressing NPC cells (Chen et al, 2003). However, no direct connection to any LMP1 signaling domain or pathway has been drawn in this respect so far. Other studies have shown that PKCδ and ERK are involved in the phosphorylation of STAT3 at Ser727 in a CTAR1-dependent manner in C33A cervix carcinoma cells and CNE1 nasopharyngeal carcinoma cells, respectively (Kung et al, 2011; Liu et al, 2008). However, it was not shown if these are direct or indirect effects of LMP1 signaling. Serine phosphorylation of STAT3 was reported to be important for its full activation and modulation of its transcriptional activity (Aggarwal et al, 2009; Andres et al, 2013), therefore it might be possible that both the indirect, cytokine-dependent activation and the PKCδ/ERK-dependent phosphorylation of STAT3 at Ser727 complement each other to induce enhanced STAT3 activity.

Moreover, the lack of STAT3 activation after crosslinking stimulation of NGFR-LMP1($Y_{384}G$) indicates that no direct activation of STAT3 via another domain does occur. It was proposed that CTAR3, which encompasses two box 1 and one box 2 motives between aa 275 – 330, binds JAK3 to activate STAT proteins (Gires et al, 1999). However, it was not clearly shown if this involved STAT1 or STAT3 activation, and another publication demonstrated that mutation of the CTAR3 domain did not abrogate activation of JAK3 or STAT3 by LMP1 in LCLs (Higuchi et al,

2002). Data presented in this thesis underline this observation and demonstrate that STAT3 activation by LMP1 does not depend on CTAR3, but on indirect signaling loops originating at CTAR2.

5.2.1 CTAR2-Related Pathways Upregulate a Cocktail of Growth Factors and Cytokines

qRT-PCR based screening was employed to identify the soluble factors that were upregulated in a CTAR2-dependent manner to induce autocrine/paracrine signaling pathways. A cocktail of seven factors was verified that were significantly upregulated after 2 h crosslinking stimulation of NGFR-LMP1wt in MEFs, but not when TRAF6 was deleted or the CTAR2 domain was mutated. These factors were M-CSF, GM-CSF, CXCL1, EREG, IL-6, LIF and FGF7. Since EBV is capable of influencing many different cell types, and has been implicated to be associated in a lot of different tumors and tissues, these results show the importance of deciphering every possibility the virus and its oncoprotein LMP1 have to contribute to cellular transformation. Understanding the specific details of LMP1-induced changes of the cellular environments can greatly aid a differentiated approach to treat EBV-related diseases in different tissues.

Some of the identified inflammatory factors are already known to be associated with EBV infection and/or were described to be upregulated after LMP1 expression. This also shows that the applied MEWF:NGFR-LMP1 system works and is a relevant tool to study LMP1 signaling. IL-6 has been the most extensively studied cytokine. Expression of IL-6 has been found in nasopharyngeal carcinomas, Hodgkin and Reed-Sternberg cells and EBV positive peripheral T cell lymphoma, and it was shown to be induced by LMP1 (Eliopoulos et al, 1997; Herbst et al, 1997; Ho et al, 1999; Huang et al, 1999; Morris et al, 2008). Different mechanisms have been proposed to regulate LMP1-induced IL-6 production. Both the NF-κB pathway and the p38/MAPK pathway have been demonstrated to be involved in IL-6 regulation, and specifically PKR has been shown to be implicated in this process (Eliopoulos et al, 1999b; Eliopoulos et al, 1997; Lin et al, 2010). The present study adds the JNK pathway as a potent activator of LMP1-induced IL-6 expression, which was shown by qRT-PCR. This result stresses again that LMP1 is capable of employing multiple mechanisms to influence its cellular environment and the fate of the EBV host cell.

CXCL1 has been found to be elevated in LMP1-positive neoplastic tissues in LMP1-transgenic mice (Hannigan et al, 2011), and GM-CSF and CXCL1 were reported to be elevated in keratinocytes after expression of LMP1 (Morris et al, 2008). However, the essential contribution of CTAR2- and TRAF6-dependent pathways for the upregulation of these cytokines has not been shown so far. This thesis demonstrates that both factors are expressed upon CTAR2-dependent induction of NF-κB and MAPK pathways. Importantly, GM-CSF was also found to be

partially responsible for the indirect activation of Akt and ERK by NGFR-LMP1 in MEFs. This suggests that LMP1 upregulates this cytokine to ensure enhanced activation of Akt and ERK in LMP1-expressing cells by autocrine signaling, but also to induce paracrine effects in surrounding tissues, aiding tumor growth through inflammation, growth stimulation and survival responses.

B cells infected with EBV were found to upregulate M-CSF (Reisbach et al, 1989), but to my knowledge there have not been any reports so far showing M-CSF as a direct target of LMP1. Interestingly, BARF1, which is expressed and secreted early during EBV latency (Seto et al, 2005), was found to be a decoy receptor for human M-CSF and thereby acts as a modulator of immune responses against EBV infection by interfering with IFNα secretion from mononuclear cells (Cohen & Lekstrom, 1999; Elegheert et al, 2012; Hoebe et al, 2012; Shim et al, 2012). It seems contradictory that LMP1 induces the expression of M-CSF while another EBV-encoded protein counteracts its function. Therefore the exact function of LMP1-induced M-CSF would have to be evaluated in further detail and possibly within an in vivo tumor environment.

EREG was reported to be constitutively upregulated by LMP1 (Charalambous et al, 2007), and LMP1-CTAR1 alone was able to marginally induce EREG in C33A cells (Kung et al, 2011), which seemingly contradicts the essential role for CTAR2 in EREG induction presented in this study. However, the study by Kung et al. did not address EREG expression after transfection of the cells with full-length LMP1, and it is possible that EREG expression mediated by CTAR1 alone falls short of full-length LMP1, and that CTAR1 is enough to produce basal cytokine production in C33A cells (Kung et al, 2011). Functionally, EREG is a potent activator of the epidermal growth factor receptor (EGFR), which itself is also induced by LMP1 and via STAT3 (Kung & Raab-Traub, 2008; Thornburg & Raab-Traub, 2007). EGFR is found highly upregulated in undifferentiated NPC tissues and correlates with a bad prognosis (Ma et al, 2003; Zheng et al, 1994), which suggests that induction of EREG by LMP1-expressing cells aids progression of the tumor. This study represents the first report on the involvement of CTAR2-dependent pathways in the upregulation of EREG. The JNK pathway was the most prominent inducer of EREG mRNA, with contributions of both p38/MAPK and canonical NF-κB. Knowledge of this signaling mechanism furthers the understanding of LMP1-driven tumor growth.

LIF and its role in LMP1-induced signaling will be discussed in more detail in chapter 5.2.2.

Taken together, the presented results clearly show the potential of CTAR2-regulated NF-κB and MAPK pathways to induce a group of cytokines and growth factors, which aid tumor formation and progression, and which are key players of tumor associated inflammation. These results will have to be translated to clinical cases of NPC or other EBV-associated diseases like HL or

PTLD. The present study also focused on indirect signaling and cytokine induction in EBV-relevant NPC cells and LCLs, which will be discussed in chapter 5.5.

5.2.2 LIF is a Novel Mediator of LMP1-CTAR2-Related Autocrine/Paracrine Signaling Effects

Production of LIF has long been associated with EBV infection, but so far it has never been shown that LMP1 directly induces this cytokine (Bertotto et al, 1983; Lo et al, 2006). The data shown in this thesis demonstrate that LMP1 potently induces LIF mRNA in a CTAR2-dependent manner. This occurred early after induction of NGFR-LMP1 in MEFs. Induction of LIF mRNA was greatly reduced after inhibition of the JNK pathway, the p38/MAPK pathway and the canonical NF-κB pathway, which demonstrates that LMP1 employs several mechanisms to upregulate this factor, and CTAR2-dependent pathway cooperate in this process. It is known that LIF can be induced by canonical NF-κB and MAPK pathways by different receptors and in different cellular systems (Fan et al, 2004; Hartner et al, 1994), but this has never been shown for LMP1 signaling before.

Importantly, neutralization of LIF in conditioned medium from NGFR-LMP1wt cells completely blocked indirect STAT3 activation and reduced activation of Akt and ERK. This result demonstrates that LIF is a central factor of autocrine/paracrine signaling by LMP1 in these cells.

So far, autocrine/paracrine activation of STAT3 in LMP1 signaling in epithelial cells has been largely attributed to IL-6 (Chen et al, 2003). However, neutralization of IL-6 only marginally reduced the autocrine/paracrine activation of STAT3 in MEFs, while LIF neutralization inhibited STAT3 phosphorylation. Both LIF and IL-6 belong to the same family of cytokines that signal via the gp130 receptor family, and they both are associated with inflammation (Heinrich et al, 2003; Silver & Hunter, 2010). Therefore it must be considered that LIF is an essential mediator of STAT3 signaling by LMP1 in addition to IL-6, and that their actions might differ with regard to the cell type. Additionally, LMP1-induced LIF might aid tumor progression by enhancing Akt and ERK activation through autocrine mechanisms. Figure 5-1 summarizes the proposed autocrine/paracrine signaling mechanism employed by LMP1-CTAR2 through upregulation of cytokines like LIF. Future studies will need to focus on the differential impact of IL-6 and LIF on various EBV-related tumor tissues or cell lines. It is likely that EBV-induced tumor progression can react dynamically on the presence of cytokines like LIF or IL-6, and that tumors from diverse tissues or in different progression stages rely on different cytokines, which can be provided by LMP1. It is mandatory to consider this for tumor treatment or drug development.

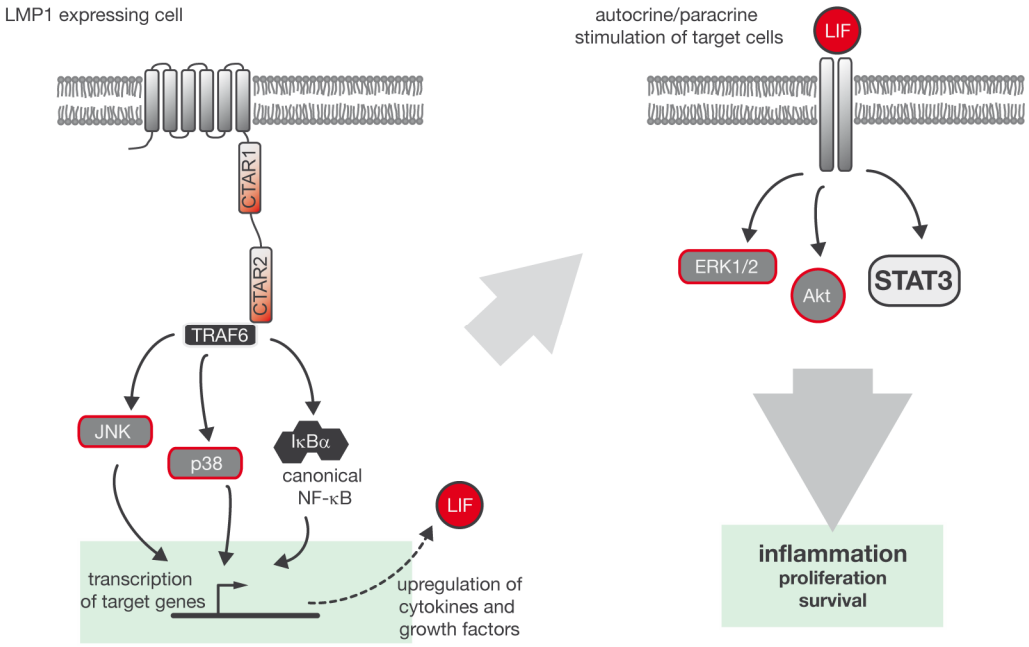

Figure 5-1. Model of LMP1-dependent autocrine/paracrine signaling mechanisms. CTAR2- and TRAF6-dependent pathways cooperate to upregulate a cocktail of cytokines and growth factors including LIF. Soluble factors are released and stimulate target cells in an autocrine/paracrine fashion. LIF was found to be the major inducer of STAT3 activation, which was entirely depending on indirect signaling mechanisms in MEFs. Both Akt and ERK can additionally be activated by LMP1-dependent indirect signaling, and LIF is involved in both pathways as well. The autocrine/paracrine activation of STAT3, Akt and ERK by LMP1 is likely to lead to inflammatory responses, but also to induce survival responses and cell proliferation.

5.3 Indirect Signaling Mechanisms in Nasopharyngeal Carcinoma Cells and LCLs

Like this thesis, many studies dealing with LMP1-induced signaling and oncogenesis have been conducted with the help of murine models, and the transforming potential of LMP1 had first been demonstrated with the help of Rat-1 fibroblasts (Moorthy & Thorley-Lawson, 1993; Wang et al, 1985). Furthermore, different in vivo carcinogenesis models demonstrated that LMP1 promoted tumor growth in transgenic mice. This was true for both lymphomas as well as transformation of epithelial cells (Kulwichit et al, 1998; Shair et al, 2012; Wilson et al, 1990; Zhang et al, 2012). Nonetheless, murine cells are not a natural target of EBV and the results gained with the MEF:NGFR-LMP1 system would have to be translated into more EBV-relevant systems. To address the question if similar indirect signaling mechanisms are employed by LMP1 in EBV target cells, some of the experimental approaches addressed in MEFs were performed in the CNE-L nasopharyngeal cell line as well as in LCLs.

5.3.1 LMP1 Indirectly Activates STAT3 in a Nasopharyngeal Carcinoma Cell Line

Expression of LMP1 in CNE-L cells confirmed STAT3 activation in these cells, although it is likely that CNE-L cells generally respond weakly to induction of STAT3. NGFR-LMP1wt also induced STAT3 activation upon crosslinking-stimulation in CNE-L cells with similar kinetics to the STAT3 phosphorylation seen in wt MEF:NGFR-LMP1wt cells. This supports the fact that LMP1 is capable of inducing STAT3 in CNE-L cells. Importantly, cycloheximide treatment during crosslinking of NGFR-LMP1wt on CNE-L cells abrogated STAT3 activation, which demonstrates that, like in MEFs, this is an exclusively indirect mechanism. Furthermore, stimulation of CNE-L cells with conditioned supernatant from LMP1wt-expressing CNE-L cells resulted in clear and enhanced activation of STAT3. In contrast, no STAT3 was phosphorylated after stimulation with supernatant from LMP1(Y_{384}G)-expressing cells. These data demonstrate that, similar to the data obtained from the MEF system, LMP1-CTAR2 is responsible for the upregulation of soluble factors, which stimulate STAT3 activation on an indirect way in a nasopharygeal carcinoma cell line.

In summary, the obtained data not only suggest an exclusively indirect, CTAR2-dependent activation mechanism for STAT3 in nasopharyngeal carcinoma cells by LMP1, but also demonstrate that LMP1 signaling mechanisms are comparable among murine fibroblasts and human NPC derived cell lines.

Unfortunately, it was not possible to evaluate indirect activation of Akt in CNE-L cells. Phosphorylated Akt was constitutively present in high levels in CNE-L cells, although FCS-depletion could reduce Akt phosphorylation. This indicates that CNE-L cells respond with heavy, sustained PI3K/Akt signaling to cytokines in the medium. Stimulation of fresh, starved CNE-L cells with conditioned supernatant from LMP1-transfected cells induced phosphorylation of Akt with no regard to LMP1wt or mutant. It is conceivable that factors, which are continuously produced by CNE-L cells, activate Akt in an autocrine/paracrine manner, and that this signal was strong enough to override signal induction via cytokines specifically upregulated by LMP1-CTAR2. The use of other, less sensitive NPC cell lines might be possible in future experiments, to help clarify the need for CTAR2-dependent autocrine/paracrine activation of Akt in NPC cells.

Similarly, ERK activation was induced by indirect signaling mechanisms, with small regard for medium conditioned by LMP1wt or LMP1 mutants. However, medium conditioned by LMPwt led to a slightly increased phosphorylation of ERK in the CNE-L target cells that medium conditioned by LMP1(Y_{384}G). This suggests that indirect activation of ERK in CNE-L cells is possible through upregulation of soluble factors via LMP1-CTAR2. Again, this demonstrated that the results obtained with the help of the MEF:NGFR-LMP1 system are transferrable to NPC cell lines.

5.3.2 Inflammatory Cytokines Are Upregulated by LMP1-CTAR2 in CNE-L Cells and LCLs

LMP1-dependent expression of selected cytokines and growth factors was investigated by qRT-PCR in both CNE-L cells and P493-6 LCLs.

Expression of constitutively active HA-tagged LMP1 as well as crosslinking-stimulation of NGFR-LMP1 in CNE-L cells resulted in the induction of a cocktail of growth factors reminiscent of that found in MEFs. Especially LIF was also a target of LMP1 in this NPC cell line, and the LIF levels after HA-LMP1 expression were comparable to IL-6, which has been reported to be a major inflammatory factor in NPC or HL (Chow et al, 2003; Herbst et al, 1997; Huang et al, 1999). This indicates that LIF might also be an important cytokine in NPC-related inflammation. Interestingly, no FGF7 or GM-CSF were found to be induced by LMP1 in CNE-L cells. This was particularly noteworthy, because GM-CSF was partially responsible for indirect activation of Akt and ERK in MEFs. However, especially the phosphorylation of Akt was deregulated in CNE-L cells, and indirect activation of this protein could not be evaluated in CNE-L cells. Possibly this specific mechanism does not play an important role in NPC cells. The importance of LIF for indirect activation of STAT3 in CNE-L cells has yet to be confirmed, but it is likely that this cytokine is at least partially responsible for STAT3 activation in NPC, possibly in concert with IL-6 (Chen et al, 2003).

The present study was the first to show LIF as a direct target of LMP1 in NPC cells. Moreover, it was possible to significantly reduce LIF induction by mutation of CTAR2. Similarly, M-CSF, CXCL1, EREG and IL-6 production were also substantially reduced when the CTAR2 domain was mutated. This demonstrates that, similar to MEFs, cytokine production on CNE-L cells was also dependent on CTAR2 induced signaling pathways. It would be interesting to learn about the contribution of the different CTAR2-induced signaling pathways to the upregulation of LIF and other cytokines and growth factors in NPC cells in the future.

Induction of cytokines was finally also tested in P493-6 LCLs, either under the control of a myc- or an EBNA2-driven growth program. Again, M-CSF, CXCL1, EREG and LIF were significantly upregulated, when LMP1 was expressed after EBNA2 activation. Interestingly, no IL-6 or GM-CSF were found upregulated in these cells, which indicates an even more important role for LIF in B cell associated transformation mechanisms. The cytokine profile in P493-6 LCLs was reminiscent of that in MEFs and CNE-L cells, but it is obvious that there were variations between the cell lines. Of course, P493-6 cells do not only express LMP1 as a consequence of EBNA2, but all latency III genes including LMP2A. Therefore cytokine production or, on the other hand, counter-regulation of this could be attributed also to LMP2A. In fact, studies have shown that LMP2A is able to modulate LMP1 induced effects, for example by downregulation of TRAF2

(Guasparri et al, 2008; Vrazo et al, 2012), and this modulatory effect might explain the slight difference in cytokine expression in P493-6 compared to MEFs and CNE-L cells. Therefore, it would be interesting to evaluate the exact contribution of LMP1, and more specifically of CTAR2, to cytokine production in LCLs, possibly by transient expression of HA-tagged LMP1 in B cell lines.

In summary, the presented data clearly show that there are striking similarities among the tested LCL and NPC cell lines with regard to their cytokine and growth factor response to LMP1, and that the found cytokine pattern was similar to that found in MEFs. Moreover, cytokine induction was also CTAR2 dependent in CNE-L cells. Still, some differences in the cytokine pattern were obvious, which likely reflect general differences among the cell types tested.

5.4 Possible Implications of Autocrine/Paracrine LMP1 Signaling for EBV-Related Diseases

Inflammation has been associated with cancer for a long time. Chronic inflammation can aid neoplastic development and significantly increases the risk to develop tumors (Grivennikov et al, 2010; Mantovani et al, 2008). In this context, cytokine-dependent activation of STAT3 in particular has always been discussed as a major tumorigenic factor, and deregulated STAT3 is often found in different tumors (Aggarwal et al, 2009; Fagard et al, 2013; Pensa et al, 2009). STAT3 supports tumor progression through different mechanisms including cell cycle progression, induction of anti-apoptotic factors and supporting angiogenesis and metastasis (Kiuchi et al, 1999; Liu et al, 2003; Masuda et al, 2002; Song et al, 2008; Wei et al, 2003). Inflammation is also typically associated with EBV-related diseases like Hodgkin's lymphoma or nasopharyngeal carcinoma (Herbst et al, 1997; Huang et al, 1999; Li et al, 2007). However, the initial triggers for these inflammatory processes are not entirely understood. It was proposed that nasopharyngeal cells are involved in maintenance and amplification of inflammation, which can ultimately lead to development of NPC, by producing pro-inflammatory cytokines in response to medium harvested from activated macrophages (Liao et al, 2012). This would induce a feedback loop which further promoted inflammatory processes involving NF-κB and STAT3 (Liao et al, 2012). Since NPC is widely associated with EBV infection, it is possible that LMP1 similarly triggers and sustains local inflammation to promote tumor development and progression. LMP1 has been shown to induce a wide range of inflammatory cytokines, which has also been addressed in this thesis. LMP1-induced cytokines like LIF cannot only affect the LMP1-expressing cells in autocrine loops, but also promote STAT3, Akt and ERK activation in neighboring cells in a paracrine way. Additionally, cells secreting inflammatory cytokines in response to LMP1 expression attract further inflammatory cells to the tumor environment,

thereby aiding a feedback mechanism, which promotes tumor development and growth. In fact, STAT3 has been proposed to be a central therapeutic target for NPC (Ho et al, 2013). Establishing LIF as a key modulator of STAT3 activation in NPC may aid development of therapeutic strategies against EBV induced tumorigenesis by targeting this signaling mechanism.

It has been further reported that LMP1 influences the expression of MMP-9 through the NF-κB and JNK pathways, thereby promoting metastasis (Stevenson et al, 2005; Yoshizaki, 2002; Yoshizaki et al, 1998). Strong expression of MMP-9 is also induced by enhanced STAT3 signaling in human mammary epithelial cells (Dechow et al, 2004). Knowing that both the canonical NF-κB and the JNK pathway are involved in LIF upregulation by LMP1, and that LIF is the prominent autocrine/paracrine activator of STAT3 in the cells tested in this thesis, it is possible that this feedback loop is capable of enhancing LMP1-mediated MMP-9 activation.

The transforming abilities of LMP1 have been ascribed to different pathways like JNK, NF-κB, Akt and ERK (Cahir McFarland et al, 1999; Dawson et al, 2003; Kutz et al, 2008; Mainou et al, 2005; Mainou et al, 2007). Indirect activation of Akt and ERK, as mentioned before, may also enhance the transforming abilities of the JNK and NF-κB pathways directly engaged by LMP1. This may not only affect LMP1-infected cells themselves, but also surrounding tissues by paracrine effects.

In the context of already published data, the results presented within this thesis therefore greatly aid our understanding of the vastness of LMP1-induced signaling networks, which drive EBV-mediated cell transformation and neoplasia.

5.5 Outlook

The results presented in this thesis demonstrated that CTAR2-dependent signaling pathways JNK, p38/MAPK and NF-κB cooperate to induce autocrine/paracrine activation of STAT3, Akt and ERK in MEFs, primarily through the cytokines LIF and GM-CSF. This was partially also true for NPC cells. This finding presents a basis for further studies aiming at finding possibilities to inhibit LMP1 and its potential to promote tumor development by targeting selected signaling mechanisms including LIF upregulation and/or STAT3 activation. Understanding that CTAR2 and TRAF6 are a common origin for such a large variety of pathways that affect cell growth and survival and ultimately lead to cancer progression, immediately suggests that the association of TRAF6 with CTAR2 presents a primary target for therapeutic intervention and inhibition. In the future it will be sensible to critically and in detail investigate the exact interaction of TRAF6 with LMP1, and its implications for LMP1 signaling, and establish this molecule as a target for therapy of EBV/LMP1-related diseases.

The TRAF6 rescue system presented in this thesis can be used as a basis to study the exact molecular role of TRAF6 in more detail. Different mutants of TRAF6 can be tested and their impact on LMP1-induced direct and indirect signaling can be studied.

Furthermore, the results presented in this thesis urge the need to revisit the role of TRADD in LMP1 signaling in more detail. Even if TRADD does not seem to be essential for CTAR2-induced signaling, it aids strong activation of all CTAR2-mediated direct and indirect signaling pathways.

Finally, slight differences in LMP1-mediated signaling mechanisms and cytokine production among different cell lines became obvious during the course of this work. Since LMP1 expression is also associated with various cell types and diseases, it may be helpful to investigate certain aspects of LMP1-induced signaling mechanisms, like TRAF6 utilization or cytokine production, in close detail in parallel studies of different cell types that are related to EBV-driven diseases. This may aid the development and advancement of more focused, specialized therapeutic approaches targeting EBV-related malignancies.

6 SUMMARY

Latent membrane protein 1 (LMP1) is the primary oncogene of Epstein-Barr Virus (EBV), and its expression is associated with several severe malignancies such as nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma or post transplant-lymphoproliferative disorders. LMP1 acts as a constitutively active, ligand-independent receptor that exploits cellular signaling pathways reminiscent of the TNF-receptor family. Like TNFR1 or CD40 in B cells, LMP1 recruits cellular signaling molecules of the TRAF family and TRADD to its two C-terminal signaling regions CTAR1 and CTAR2. To exert its oncogenic potential, and to efficiently transform different cell types in vivo and in vitro, LMP1 engages NF-κB and MAPK pathways, as well as the PI3K/Akt pathway. Furthermore, LMP1 contributes to neoplasia and tumor growth by mediating STAT3 activation and the induction of inflammatory cytokines like IL-6. Although extensive research has focused on the signal transduction mechanisms employed by LMP1, some aspects still remain incompletely understood or controversial.

This thesis aimed at clarifying some of these aspects. In particular, the contributions of CTAR1 and CTAR2 as well as cellular adapter proteins to signal transduction was studied with the help of mutated LMP1 receptors, which were stably expressed in mouse embryonic fibroblasts (MEFs) deficient for TRAF6, TRADD and both TRAF2 and TRAF5. Inducible chimeric NGFR-LMP1 receptors, which were activated by antibody crosslinking, were favored over native, constitutively active LMP1. This allowed examination of LMP1-dependent signal induction in a

time-dependent manner with a distinct induction point, and provided the advantage to discriminate direct from late, indirect signaling events. Together the MEF:NGFR-LMP1 system offered the opportunity to study LMP1-dependent signaling mechanisms and networks in a comprehensive, systematical way, and proved to be a powerful tool to do so.

The results presented in this thesis demonstrate a central role for the CTAR2 domain and the adaptor protein TRAF6 in various signaling pathways. They confirm an essential contribution of CTAR2 and TRAF6 to the induction of the JNK, p38 and canonical NF-κB pathway. Furthermore, a critical dependence of LMP1-induced STAT3, Akt and ERK activation on CTAR2 and TRAF6 was shown here for the first time. The death domain protein TRADD was further demonstrated to be involved in all of these pathways in a non-essential but contributing manner.

The use of inducible NGFR-LMP1 receptors rendered experiments possible, which helped uncover important, indirect signaling mechanisms employed by LMP1. Strong, persistent activation of both Akt and ERK was revealed to rely on indirect signaling mechanisms involving the CTAR2- and TRAF6-dependent upregulation of soluble factors. It was also shown for the first time that activation of STAT3 by LMP1 exclusively relied on indirect, autocrine/paracrine signaling mechanisms mediated by CTAR2 and TRAF6. Cooperation of canonical NF-κB, JNK and p38 signaling led to the upregulation of a cocktail of cytokines and growth factors by LMP1, among which leukemia inhibitory factor (LIF) was identified as a novel target of CTAR2-dependent LMP1 signaling. In addition, LIF was demonstrated to be almost exclusively responsible for indirect STAT3 activation by NGFR-LMP1 in MEFs, while both LIF and GM-CSF mediated indirect phosphorylation of Akt and ERK.

STAT3 activation and inflammatory cytokines play an especially important role for the progression of certain EBV-associated tumors like nasopharyngeal carcinoma (NPC). Data presented in this thesis demonstrate that LMP1-dependent activation of STAT3 is mediated by soluble factors in nasopharyngeal carcinoma cells. Furthermore, a similar cocktail of cytokines and growth factors, including LIF, was identified to be upregulated after LMP1 expression in a CTAR2-dependent manner in NPC cells. Finally, the induction of cytokines and growth factors was also demonstrated in LCLs, and especially LIF was significantly increased in these cells as well, indicating that LIF is an important, novel effector and mediator of LMP1 functions in several cell types.

7 ZUSAMMENFASSUNG

Das latente Membranprotein 1 (LMP1) ist das wichtigste Onkogen des Epstein-Barr Virus (EBV) und seine Expression steht in Verbindung mit verschiedenen malignen Erkrankungen wie Nasopharynxkarzinomen (NPC), Hodgkin-Lymphomen oder Posttransplantationslymphomen. LMP1 wirkt wie ein konstitutiv aktiver, ligandenunabhängiger Rezeptor, der zelluläre Signalwege anregt, die auch bei den Rezeptoren der TNF-Rezeptorfamilie zu finden sind, und rekrutiert, ähnlich wie TNFR1 oder CD40, zelluläre Signalmoleküle der TRAF Familie oder TRADD über die beiden C-terminalen Regionen CTAR1 und CTAR2. Für die onkogene Wirkung, und um verschiedene Zellarten sowohl in vivo als auch in vitro effizient zu transformieren, aktiviert LMP1 NF-κB und MAPK Signalwege ebenso wie den PI3K/Akt Signalweg. Außerdem trägt LMP1 zu Neoplasie und Tumorwachstum bei, indem es die Aktivierung von STAT3 vermittelt und die Freisetzung inflammatorischer Zytokine wie IL-6 induziert. Obwohl es zahlreiche Studien zu den Mechanismen der Signalweiterleitung durch LMP1 gibt, bleiben zahlreiche Aspekte nach wie vor unvollständig geklärt oder kontrovers.

Die vorliegende Arbeit hatte zum Ziel einige dieser Aspekte zu beleuchten und aufzuklären. Genauer sollte der Beitrag sowohl der beiden Domänen CTAR1 und CTAR2 als auch bestimmter zellulärer Adapterproteine, sowie deren Zusammenspiel untersucht werden. Hierzu wurden mutierte LMP1-Rezeptoren in verschiedenen embryonalen Mausfibroblasten (MEF), denen TRAF6, TRADD oder TRAF2 und TRAF5 fehlen, stabil exprimiert. Statt nativer,

konstitutiv aktiver LMP1-Rezeptoren wurden induzierbare, chimäre NGFR-LMP1 Rezeptoren vorgezogen, die durch antikörpervermittelte Kreuzvernetzung aktiviert werden konnten. Diese Methode erlaubte die Untersuchung der LMP1-induzierten Signalwege in Abhängigkeit der Aktivierungszeit mit einem definierten Startpunkt, mit dem Vorteil, direkte von indirekten Signalen unterscheiden zu können. Somit ermöglichte das MEF:NGFR-LMP1 System, umfassende, systematische Untersuchungen LMP1-abhängiger Signalmechanismen und Netzwerke, und erwies sich als überzeugendes Werkzeug für derartige Studien.

Die Ergebnisse der vorliegenden Studie zeigen, dass die CTAR2-Domäne und das Signalmolekül TRAF6 eine zentrale Rolle in verschiedenen Signalwegen spielen. Es konnte bestätigt werden, dass CTAR2 und TRAF6 essenziell zur Aktivierung der JNK-, p38- und NF-κB-Signalwege beitragen. Außerdem ließ sich beobachten, dass die LMP1-induzierte Aktivierung von STAT3, Akt und ERK ebenfalls kritisch von CTAR2 und TRAF6 abhängig ist. Zusätzlich wurde gezeigt, dass das Todesdomänenprotein TRADD ebenfalls einen Beitrag in all diesen Signalwegen leistet, allerdings keine essenzielle Rolle spielt.

Durch die Induzierbarkeit der NGFR-LMP1-Rezeptoren waren Experimente möglich, durch die gezeigt werden konnte, dass wichtige Signalmechanismen von LMP1 indirekt angesteuert werden. Es wurde klar, dass eine starke, anhaltende Aktivierung von Akt und ERK auf indirekte Signalmechanismen angewiesen ist, welche die Hochregulierung löslicher Faktoren durch CTAR2- und TRAF6-abhängige Mechanismen einschließen. Ebenso konnte zum ersten Mal gezeigt werden, dass die Aktivierung von STAT3 durch LMP1 ausschließlich auf indirekten, autokrinen/parakrinen Mechanismen beruht, die ebenfalls durch CTAR2 und TRAF6 vermittelt werden. Das Zusammenspiel von JNK, p38 und kanonischem NF-κB führte zur Hochregulierung verschiedener Zytokine und Wachstumsfaktoren durch LMP1, unter denen der Leukämie-inhibierende Faktor (LIF) als neues Zielprotein von LMP1-CTAR2-abhängigen Signalwegen identifiziert wurde. Zusätzlich konnte gezeigt werden, dass LIF beinahe eigenständig für die indirekte Aktivierung von STAT3 durch NGFR-LMP1 in MEF Zellen verantwortlich war, während die indirekte Aktivierung von Akt und ERK sowohl durch LIF als auch durch GM-CSF vermittelt wurde.

Die Aktivierung von STAT3 und die Ausschüttung inflammatorischer Zytokine spielen besonders bei der Ausbildung und Tumorgenese EBV-assoziierter Tumore wie dem Nasopharynxkarzinom (NPC) eine entscheidende Rolle. Die in dieser Arbeit vorgestellten Daten zeigen, dass die STAT3-Aktivierung durch LMP1 in NPC-Zelllinien ebenfalls durch lösliche Faktoren vermittelt wird. In diesen Zellen wird der Zytokincocktail, der dem in MEF Zellen identifizierten sehr ähnlich ist, ebenfalls durch LMP1-CTAR2-abhängige Signalwege freigesetzt, und auch hier konnte die Hochregulierung von LIF nachgewiesen werden. Schließlich konnte die LMP1-abhängige

Induktion der Zytokine und vor allem von LIF auch in LCLs gezeigt werden. Diese Befunde deuten darauf hin, dass LIF ein wichtiger, neuer Effektor und Mediator für LMP1-abhängige Funktionen in verschiedenen Zelltypen ist.

8 APPENDIX

8.1 Amino acids

G	Gly	Glycine
A	Ala	Alanine
S	Ser	Serine
T	Thr	Threonine
C	Cys	Cysteine
V	Val	Valine
L	Leu	Leucine
I	Ile	Isoleucine
M	Met	Methionine
P	Pro	Proline
F	Phe	Penylalanine
Y	Tyr	Tyrosine
W	Trp	Tryptophan
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
N	Asn	Asparagine
Q	Gln	Glutamine
H	His	Histidine
K	Lys	Lysine
R	Arg	Arginine

8.2 Abbreviations

°C	degrees celcius
µg	microgram
µl	microliter
aa	amino acid
BL	Burkitt's lymphoma
BSA	bovine serum albumin
CD	C-terminal domain
CD	cluster of differentiation
CFP	cyan fluorescent protein
cIAP	cellular inhibitor of apoptosis
cm	centimeter
CSF	colony stimulating factor
CTAR	C-terminal activation region
CXCL	chemokine ligand (C-X-C motif)
DNA	deoxyribonucleic acid
dNTP	deoxynuceotide
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EREG	epiregulin
ERK	extracellular-signal regulated kinase
FACS	fluorescence activated cell sorting, flowcytometry
FCS	fetal calf serum
FGF	fibroblast growth factor
fwd	forward
GFP	green fluorescent protein
h	hour
HA	hemagglutinin
HL	Hodgkin's lymphoma
HRP	horseradish peroxidase
IκB	Inhibitor of kappa B
IKK	IκB kinase
IL	Interleukin
IM	infectious mononucleosis
IRES	internal ribosomal entry site
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
l	liter
LCL	lymphoblastoid cell line
LIF	leukemia inhibitory factor
LMP	latent membrane protein
LTR	long terminal repeats
M	molar
mA	milliampere
MAPK	mitogen activated protein kinase
MEF	mouse embryonic fibroblast
MFI	mean fluorescence intensity

min	minute
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B cells
ng	nanogram
NGFR	nerve growth factor receptor
NIK	NF-κB inducing kinase
NL	NGFR-LMP1
nm	nanometer
NPC	nasopharyngeal carcinoma
PCR	polymerase chain reaction
PI3K	phosphatidylinositide 3-kinase
pmol	picomol
PTLD	posttransplant lymphoproliferative disorder
q	quantitative
rev	reverse
(m)RNA	(messenger) ribonucleic acid
rpm	rotations per minute
RT	real time
RT	reverse transcriptase
RT	room temperature
SD	standard deviation
sec	second
STAT	signal transducer and activator of transcription
TIR	Toll like / IL-1 receptor
TLR	Toll-like receptor
TM	transmembrane domain
TNF	tumor necrosis factor
TRADD	TNF receptor associated death domain protein
TRAF	TNF receptor associated factor
U	Units
V	volt
v/v	volume per volume
w/v	weight per volume
wt	wildtype
x g	multiples of gravity

8.3 Qiagen RT^2 Profiler PCR Array (PAMM-041F) targets (functional gene groupings)

8.3.1 Angiogenic Growth Factors

BMP4	bone morphogenetic protein 4
EREG	epiregulin

FGF1	fibroblast growth factor 1
FGF2	fibroblast growth factor 2
FGF6	fibroblast growth factor 6
FIGF	c-Fos induced growth factor
PDGFa	platelet derived growth factor alpha
PGF	placental growth factor
PTLD	post-transplant lymphoproliferative disorder
TGFa	transforming growth factor alpha
VEGFa	vascular endothelial growth factor A
VEGFb	vascular endothelial growth factor B
VEGFc	vascular endothelial growth factor C

8.3.2 Apoptosis regulators

BDNF	brain derived neurotrophic factor
GDF5	growth differentiation factor 5
GDNF	glial cell line derived neurotrophic factor
IGF1	insulin-like growth factor 1
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-7	Interleukin 7
RABEP1	rabaptin, RAB GTPase binding effector protein 1
SPP1	secreted phosphoprotein 1
VEGFa	vascular endothelial growth factor A

8.3.3 Cell differentiation

BDNF	brain derived neurotrophic factor
BMP1	bone morphogenetic protein 1
BMP2	bone morphogenetic protein 2
BMP3	bone morphogenetic protein 3
BMP4	bone morphogenetic protein 4
BMP5	bone morphogenetic protein 5
BMP6	bone morphogenetic protein 6
BMP7	bone morphogenetic protein 7
BMP8a	bone morphogenetic protein 8a
BMP8b	bone morphogenetic protein 8b
M-CSF (CSF1)	colony stimulating factor 1 (macrophage)
GM-CSF (CSF2)	colony stimulating factor 2 (granulocyte-macrophage)
EREG	epiregulin
FGF1	fibroblast growth factor 1
FGF10	fibroblast growth factor 10
FGF2	fibroblast growth factor 2
FGF5	fibroblast growth factor 5
FGF6	fibroblast growth factor 6

FGF9	fibroblast growth factor 9
FIGF	c-Fos induced growth factor
GDF11	growth differentiation factor 11
IGF1	insulin-like growth factor 1
IL-4	Interleukin 4
IL-7	Interleukin 7
LEP	leptin
Mdk	midkine
NTF3	neurotrophin 3
NTF5	neurotrophin 5
PGF	placental growth factor
SPP1	secreted phosphoprotein 1
TFF1	trefoil factor 1
VEGFa	vascular endothelial growth factor A
VEGFb	vascular endothelial growth factor B
VEGFc	vascular endothelial growth factor C
ZFP91	zinc finger protein 91

8.3.4 Development controllers

AMH	anti-mullerian hormone
ARTN	artemin
BMP1	bone morphogenetic protein 1
BMP10	bone morphogenetic protein 10
BMP2	bone morphogenetic protein 2
BMP3	bone morphogenetic protein 3
BMP4	bone morphogenetic protein 4
BMP5	bone morphogenetic protein 5
BMP6	bone morphogenetic protein 6
BMP7	bone morphogenetic protein 7
BMP8a	bone morphogenetic protein 8a
BMP8b	bone morphogenetic protein 8b
CXCL12	chemokine ligand (C-X-C motif) 12
EREG	epiregulin
FGF1	fibroblast growth factor 1
FGF10	fibroblast growth factor 10
FGF15	fibroblast growth factor 15
FGF18	fibroblast growth factor 18
FGF2	fibroblast growth factor 2
FGF3	fibroblast growth factor 3
FGF6	fibroblast growth factor 6
FGF8	fibroblast growth factor 8
FGF9	fibroblast growth factor 9
FIGF	c-Fos induced growth factor
GDF11	growth differentiation factor 11
GDNF	glial cell line derived neurotrophic factor
IGF1	insulin-like growth factor 1

KITL	kit ligand
LEFTY1	left-right determination factor 1
LIF	leukemia inhibitory factor
MDK	midkine
NGFb	nerve growth factor beta
NODAL	nodal
NTF3	neurotrophin 3
NTF5	neurotrophin 5
PGF	placental growth factor
S100a6	S100 calcium binding protein A6 (calcyclin)
TGFB1	transforming growth factor beta 1
TGFB2	transforming growth factor beta 2
TGFB3	transforming growth factor beta 3
VEGFa	vascular endothelial growth factor A
VEGFb	vascular endothelial growth factor B
VEGFc	vascular endothelial growth factor C
ZFP91	zinc finger protein 91

8.3.5 Morphogenic factors

BDNF	brain derived neurotrophic factor
BMP1	bone morphogenetic protein 1
BMP2	bone morphogenetic protein 2
BMP7	bone morphogenetic protein 7
EGF	epidermal growth factor
FGF10	fibroblast growth factor 10
FGF4	fibroblast growth factor 4
FGF8	fibroblast growth factor 8
FGF9	fibroblast growth factor 9
GDF11	growth differentiation factor 11
GDF5	growth differentiation factor 5
HGF	hepatocyte growth factor
IGF1	insulin-like growth factor 1
IGF2	insulin-like growth factor 2
LIF	leukemia inhibitory factor
PDGFa	platelet derived growth factor alpha
TGFb1	transforming growth factor beta 1
TGFb3	transforming growth factor beta 3
VEGFc	vascular endothelial growth factor C
ZFP91	zinc finger protein 91

Other growth factors

G-CSF (CSF3)	colony stimulating factor 2 (granulocyte)
CXCL1	chemokine ligand (C-X-C motif) 1
FGF11	fibroblast growth factor 11

FGF13	fibroblast growth factor 13
FGF14	fibroblast growth factor 14
FGF17	fibroblast growth factor 17
FGF22	fibroblast growth factor 22
FGF7	fibroblast growth factor 7
GDF10	growth differentiation factor 10
GDF8	growth differentiation factor 8
IL-11	Interleukin 11
IL-12a	Interleukin 12A
IL-18	Interleukin 18
IL-1a	Interleukin 1 alpha
IL-1b	Interleukin 1 beta
IL-2	Interleukin 2
IL-3	Interleukin 3
INHa	inhibin alpha
INHba	inhibin beta-A
INHbb	inhibin beta-B
LEFTY2	left-right determination factor 2
TDGF1	teratocarcinoma-derived growth factor 1

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- Loriot

LIST OF PUBLICATIONS

Peer-reviewed Publications:

Vogel S, Kiefer T, Raulf N, Sterz K, Manoharan A, Borner C (2009) Mechanisms of Bax/Bak activation: Is there any light at the end of the tunnel? *Gastroenterology and Hepatology From Bed to Bench* **2**: 11-18

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Meetings:

September 2008	16th Euroconference on Apoptosis and 5th Swiss Apoptosis Meeting, Bern CH Posterpresentation: *How does PUMA regulate IL-3 deprivation-induced apoptosis?*
November 2011	15th Joint Meeting der Signal Transduction Society (STS), Weimar Posterpresentation: *Comprehensive Analysis of LMP1 signaling reveals a critical role for TRAF6 and TRADD in the activation of the PI3K/Akt pathway by LMP1*
November 2012	16th Joint Meeting der Signal Transduction Society (STS), Weimar Oral Presentation: *The EBV oncogene LMP1 requires TRAF6 to induce a cocktail of growth factors and cytokines that mediates robust PI3K/Akt activation*
November 2013	17th Joint Meeting der Signal Transduction Society (STS), Weimar Oral Presentation: *The Epstein-Barr Virus oncogene LMP1 induces STAT3 via TRAF6-dependent upregulation of the cytokine LIF*