MOLECULAR MECHANISMS CONTROLLING LYMPHOCYTE PROLIFERATION AND SURVIVAL

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The human body is exposed daily to potentially harmful infectious and non-infectious agents, and has therefore developed efficient defense systems. Lymphocytes are cells of the immune system that play a prime role in the defense against infectious agents, but also contribute to the efficient elimination of malignant cells. Lymphocytes become activated upon detection of molecular changes associated with infection or malignancy, and subsequently undergo a clonal expansion that serves to multiply a subset of cells that are most suited to eliminate the detected infectious agent or tumor cell. Subsequently, lymphocyte numbers retract, and excessive lymphocytes die because of both, a lack of sufficient growth factors in their environment and an active, ligand-induced mechanism of cell death induction (**Fig. 1**). The remaining surviving lymphocytes, also called memory lymphocytes, provide efficient protection of the body upon a later infection by the same infectious agent.

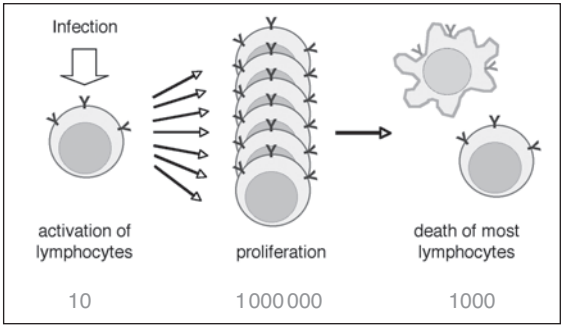

Figure 1: Lymphocyte expansion and elimination during a normal immune response. *Upon recognition of an infectious agent by the lymphocyte's cell surface receptor, lymphocytes get activated and expand approximately 100.000 fold within few days. Subsequently to the elimination of the infectious agent, most of the lymphocytes die and only a limited number of so-called memory lymphocytes remain, which protect the individual against a subsequent infection by the same pathogen.*

It is the correct control of every step in the process of activation, expansion and subsequent elimination of lymphocytes that assures an efficient adaptive immune response. Deregulation of the activation step leads to immunodeficiency, while inefficient elimination of reactive lymphocytes has been associated with autoimmune disease and development of lymphocyte-derived cancers, such as lymphoma and leukemia. Moreover, persistent activation of lymphocytes by grafted tissues, recognized by the immune system as a foreign and thus potentially harmful agent, is responsible for the rejection of organ transplants (**Fig. 2**).

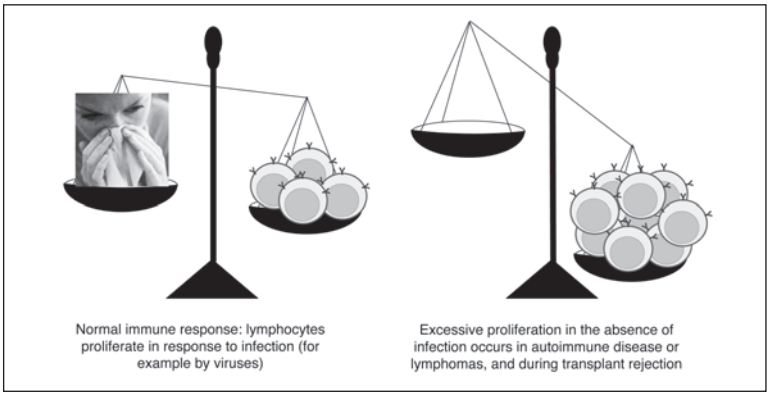

Figure 2: Consequence of excessive lymphocyte proliferation. *Normally, lymphocytes proliferate in response to infection, but excessive lymphocytes are subsequently eliminated by cell death. Excessive lymphocyte proliferation in the absence of infection can cause autoimmune disease or lead to lymphoid malignancies such as leukemia or lymphomas. Exaggerated lymphocyte responses are also responsible for the rejection of transplanted organs.*

Several lines of evidence support the need for further research on mechanisms controlling normal and pathological lymphocyte activation. On the one hand, there is a dramatic increase in autoimmune diseases worldwide, and the number of organ transplantations is continually rising. There is thus an increased need for effective and well-tolerated immunosuppressive strategies. On the other hand, a better understanding of signaling pathways leading to aberrant lymphocyte proliferation will help in the treatment of leukemias and lymphomas.

Here, I will review some of the key features that control lymphocyte activation and survival, with a special focus on lymphocyte cell surface receptors and intracellular signaling cascades that are initiated upon triggering of these receptors, and that we have studied in detail over the last thirteen years.

Steps controlling the activation and proliferation of lymphocytes

Every lymphocyte carries at its cell surface a unique receptor, the antigen receptor, which is capable of recognizing foreign antigens. Foreign or non-self antigens can be distinguished from self-antigens based on their molecular structure, and are recognized as foreign because of a prior developmental selection that allows only those lymphocytes to mature who do not have an antigen receptor cross-reacting with self-antigens, and because of additional mechanisms of peripheral tolerance that suppress autoimmunity in specific tissues (1, 2). Triggering of the antigen receptor initiates a cascade of intracellular signaling events that lead to the activation of lymphocytes, which multiply and participate actively in the immune response. So-called B-lymphocytes do so by producing protective antibodies, while another subset of lymphocytes, the T-lymphocytes, help B-lymphocytes or react by direct killing of infected or tumor cells. The signaling cascade that controls these lymphocyte responses co-ordinates many types of cellular reactions (**Fig. 3**).

Antigen receptor-induced cellular reactions include changes in the shape of the cell that allow the lymphocyte to attach tightly to the antigen-presenting cell, changes in cell metabolism required for rapid proliferation, but also changes in the expression of sets of genes that control the differentiation and survival of the activated lymphocyte. Alterations in cellular shape are linked to changes in the actin cytoskeleton that are triggered by antigen recognition. How the antigen receptor controls the actin cytoskeleton is the subject of intense research efforts (3). Most of these focus on proteins of the WASP/WAVE family and their binding partners, which promote the incorporation of soluble actin monomers into filamentous strands of actin by the Arp2/3 complex (4). Such newly forming actin filaments are thought to push

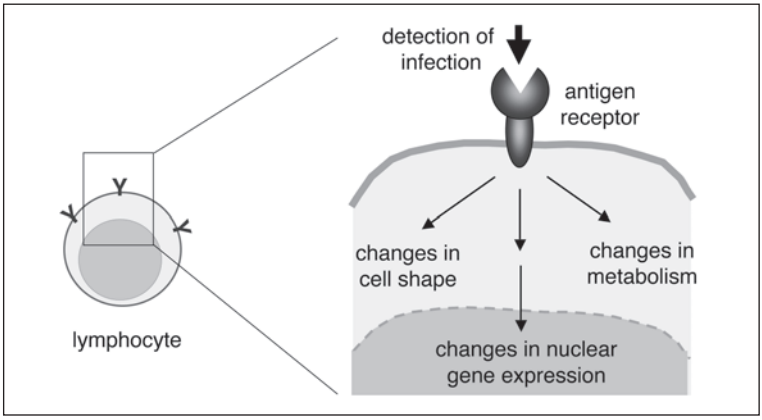

Figure 3: Cellular changes induced by detection of infection through the antigen receptor. *Triggering of the receptor leads to molecular signaling pathways (indicated by arrows) that control changes in the shape, metabolism and gene expression of the activated lymphocyte.*

membrane protrusions forward and to thereby extend the cell surface. This process is important to allow the T-cell to make efficient contacts with antigen-presenting cells, and can be mimicked *in vitro* by allowing T-lymphocytes to form conjugates with antigen-presenting cells (APCs), or to spread out on plates that are coated with agonistic T-cell receptor antibodies (5). When studying T-lymphocytes that are deficient in the protein BCL10, we observed that these cells were impaired in their capacity to form T-cell/APC conjugates or to spread out on anti-TCR coated plates (6). This was linked to a nearly total absence of T-cell receptor-induced actin polymerization (6). How exactly BCL10 controls actin polymerization is not yet understood; it is likely that BCL10 acts upstream in the signaling pathway leading from T-cell receptor engagement to the activation of WASP/WAVE family proteins, but further work is required to assess the exact contribution of BCL10. Most of our recent work has however focused on the role of BCL10 and its binding partners, CARMA1 and MALT1, in the T-cell receptor-induced transcription of genes that are relevant to lymphocyte proliferation and which will be discussed in detail below.

T-cell receptor induced changes in gene expression are controlled by transcription factors that bind specific target sequences in the genome and thereby increase or inhibit the transcription of sets of target genes (7). Two transcription factors are particularly important for lymphocyte activation: the nuclear factor of activated T-cells (NFAT) and the nuclear factor kappa B (NF-κB), which coordinately regulate the expression of genes such as interleukin-2, a cytokine that promotes T-lymphocyte proliferation *in vitro* (8). Because of their pivotal role in lymphocyte activation, both NFAT and NF-κB activating pathways are attractive targets for therapeutic immuno-modulation. The signaling events activating the transcription factors NFAT and NF-κB have been intensively studied during the last two decades. Both transcription factors are kept in an inactive form in the cytoplasm of resting cells, and translocate into the nucleus upon antigen receptor triggering, but the underlying molecular mechanisms controlling nuclear import of NFAT and NF-κB are distinct (**Fig. 4**).

In resting T-lymphocytes, NFAT is kept in the cytoplasm in an inactive, highly phosphorylated form that cannot enter the nucleus. Antigen receptor triggering induces a rise in the concentration of intracellular calcium that leads to activation of the calcium dependent Ser/Thr phophatase Calcineurin. The resulting de-phosphorylation of NFAT leads to exposure of its nuclear localization site and nuclear import of NFAT to induce transcription of genes (9, 10). NFAT-dependent gene transcription is pivotal to the adaptive immune response, and targeting the NFAT pathway is presently one of the most efficient immunosuppressive strategies. Inhibition of NFAT activation is achieved by treatment with Cyclosporin A, a compound that can enter the cell and bind to its cytoplasmic binding protein Cyclophilin. The Cyclosporin A/Cyclophilin complex in turn binds and inhibits the phosphatase Calcineurin (9). Cyclosporine and its derivatives are used to prevent transplant rejection and graft versus host disease, but also to treat certain severe forms of auto-immune diseases and immune-mediated skin diseases.

The activity of the transcription factor NF-κB is regulated by another series of signaling events that are triggered by antigen receptor engagement (11, 12). NF-κB is kept in the cytoplasm through binding to its inhibitor, the inhibitor of kappaB (IκB), and requires the proteolytic

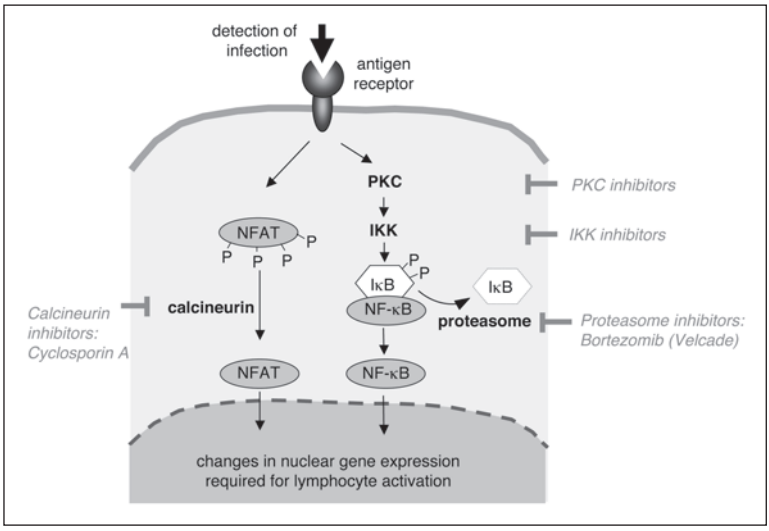

Fig. 4: Summary of key signaling events that control NFAT- and NF-κB dependent gene transcription in antigen receptor-triggered lymphocytes. *Antigen receptor triggering activates the transcription factor NFAT through its dephosphorylation, mediated by the phosphatase calcineurin. Calcineurin inhibitors, such as cyclosporine A, efficiently dampen lymphocyte-dependent immune responses and are thus used to prevent transplant rejection or treat certain auto-immune or inflammatory diseases. Therapeutic targeting of the NF-κB pathway has recently gained interest through the identification of enzymatic activities that control key steps in the pathway. These include the proteolytic activity of the proteasome, required to degrade the NF-κB inhibitor IκB, and the kinase activity of PKC family members and of the IκB kinase (IKK) complex.*

degradation of IκB by the proteasome to allow its nuclear entry. This critical step is controlled through receptor-mediated activation of the IκB kinase (IKK) complex, which phosphorylates IκB and thereby targets it for ubiquitination-dependent degradation (13). Many laboratories including ours have studied the signaling events that mediate the antigen-receptor-induced activation of IKK. One of the earliest events upon antigen receptor engagement is the activation of tyrosine kinases, which in turn activate Ser/Thr kinases of the PKC family, PKCθ (in T-cells) and PKCθ (in B-cells) that are required for IKK activation. Over the last 10 years, we and others have focused on the identification and

molecular characterization of molecular compounds controlling the NF-κB pathway downstream of PKC and upstream of IKK. This has led to the identification of the CBM complex, a protein complex comprising the proteins CARMA1, BCL10 and MALT1 which plays a central role in antigen receptor-induced NF-κB activation downstream of PKC and upstream of the IKK complex (**Fig. 5**) (11).

Through combined bioinformatic, biochemical and genetic approaches, we have identified CARMA1 as a scaffold protein that mediates NF-κB activation through recruitment of the adaptor protein BCL10 (14, 15), and that is essential for lymphocyte activation and proliferation (16). BCL10 in turn binds constitutively to the signaling protein MALT1, and it is thought that a major role of the assembled CARMA1-BCL10-MALT1 (CBM) complex is to mediate IKK activation via additional proteins such as the ubiquitin ligase TRAF6 and the Ser/Thr kinase TAK1 that control the activity of the IKK complex through its ubiquitination and phosphorylation, respectively (17). Recently, we and others could show that MALT1 is not only an adaptor protein, but that it has an enzymatic (proteolytic) activity that is essential for optimal lymphocyte activation (18, 19) (**Fig. 5**). Using a fluorogenic MALT1 substrate, we could show that MALT1 activity peaks 30 min after antigen receptor stimulation and returns to baseline levels after approximately one hour (19). This suggests that MALT1 activity is tightly regulated by unknown molecular mechanisms that are the focus of ongoing studies. Through the development of a cell-permeable inhibitor for MALT1, we could show that the protease activity of MALT1 is required for optimal NF-κB activation and cytokine production of activated T-cells (19). In addition, we found that MALT1-dependent cleavage of BCL10 affects the adhesion of T-cells to extracellular matrix proteins or cellular adhesion molecules (19), which might facilitate the binding of T-cells to antigen-presenting cells or to the endothelium of blood vessels in inflamed tissues during lymphocyte extravasation (20, 21).

With the identification of essential enzymatic activities controlling the NF-κB pathway, strategies to therapeutically interfere with NF-κB activation in lymphocytes have started to emerge (**Fig. 4 and 5**). Proteasomal inhibitors such as Bortezomib (Velcade) block NF-κB

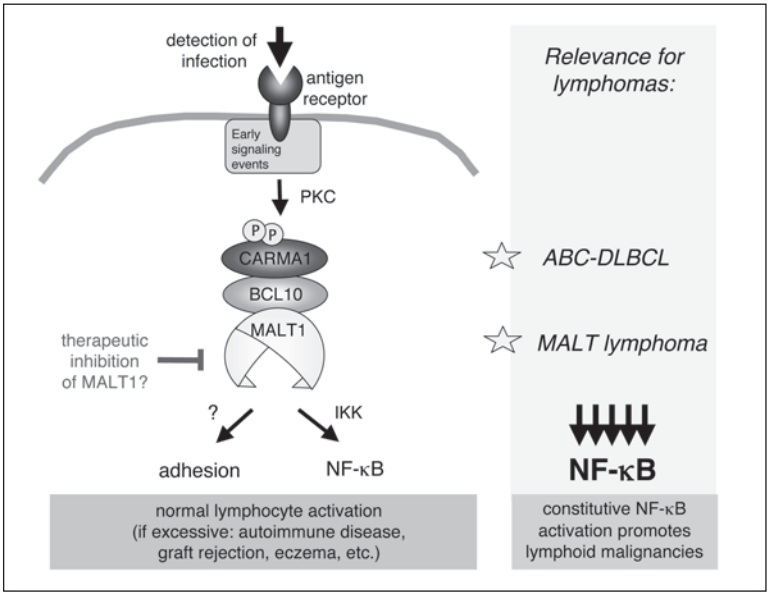

Fig. 5: Role of CBM proteins in lymphocyte activation. *Antigen receptor engagement triggers early signaling events that lead to activation of kinases of the PKC family. The proteins CARMA1, BCL10 and MALT1 control NF-κB activation upstream of the IKK complex, but also affect T-cell adhesion via unknown mechanisms. MALT1 has protease activity that is essential for both, adhesion and NF-κB activation. Therapeutic inhibition of MALT1 might be useful to treat diseases with exaggerated lymphocyte activaton, such as autoimmundiseases or eczema, or to prevent the rejection of organ transplants by activated lymphocytes. Oncogenic mutations in genes encoding CBM proteins have been reported in MALT lymphoma and the activated B-cell (ABC) subtype of diffuse large B-cell lymphoma (DLBCL), suggesting a possible further application for MALT1 inhibitors.*

activation in lymphocytes but also in many other cells, and also interfere more generally with proteasomal protein degradation. Therefore, proteasome inhibitors have toxic side effects that prevent their use for immuno-suppression, but still allow for the treatment of hematopoietic malignancies such as multiple myeloma, which is characterized by constitutive NF-κB activation (22). Similar problems have to be considered when thinking of possible applications for IKK inhibitors, which inhibit NF-κB activation in all cell types. Nevertheless, IKK inhibitors

are now in preclinical trials for immunosuppression and cancer therapy (23, 24). Since MALT1 deficiency preferentially affects adaptive immune responses (25), inhibition of MALT1 might become an interesting approach for inhibition of NF-κB activation specifically in lymphocytes, either for immuno-suppression or to treat lymphoid malignancies characterized by constitutive MALT1 activation (see below).

Uncontrolled lymphocyte proliferation and lymphoma development

Several lines of evidence support the idea that aberrant NF-kB activation in lymphocytes is associated with the development of lymphomas (26, 27). At least two types of lymphoma have been directly linked to mutations in the genes encoding CARMA1, BCL10 or MALT1 (**Fig. 5**): diffuse large B-cell lymphoma (DLBCL), which is the most common form of lymphoma in adults, and lymphomas of the mucosa-associated lymphoid tissue (so-called MALT lymphomas), a rather rare form of lymphoma (27, 28). DLBCL can be classified into at least three subtypes based on the gene expression profile (27). The so-called activated B-cell (ABC) subtype of DLBCL is characterized by constitutive activation of NF-kB and high levels of expression of NF-kB target genes (27). It was shown that silencing of the expression of CARMA1, BCL10 or MALT1 leads to impaired survival of cell lines derived from ABC-DLBCL (29), suggesting that the CARMA1-BCL10-MALT1 pathway is responsible for constitutive NF-kB activation in this type of lymphoma. Subsequently, oncogenic mutations in CARMA1 have been identified in patients with ABC-DLBCL, identifying CARMA1 as a bona fide oncogene (30). Using cell lines derived from patients with ABC DLBCL, we could recently show that MALT1 is constitutively active in these cell lines, and that MALT1 activity can be induced by oncogenic mutants of CARMA1 (Hailfinger et al., in press). Interestingly, the proliferation and survival of ABC DLBCL cell lines in vitro was strongly impaired by treatment with a MALT1 inhibitor, suggesting that MALT1 protease activity could be a disease-specific drug target (Hailfinger et al., in press). Further work is required to assess whether MALT1 protease activity is also relevant for the growth of MALT lymphomas and potentially of other malignancies associated with constitutive NF-κB activation.

Mechanisms that limit the survival of proliferating lymphocytes

The antigen receptor-induced NF-κB activation in lymphocytes is usually transient, and allows lymphocytes to proliferate and survive during a limited window of time of a few days. This assures the transient NF-κB-dependent upregulation of gene products such as cytokines, that act as growth factors to promote cellular growth and proliferation, but also of proteins that transiently make the cells resistant to cell death, such as Bcl-2 and FLIP family proteins. During the retraction phase of the immune response, the level of expression of these proteins drops down, and as a result, the cells become susceptible to cell death. This inherently programmed form of cell death of lymphocytes, also called activation-induced cell death (AICD) is thought to be the result of both, growth factor deprivation and an active fratricide-dependent mechanism of cell death induction through the death receptor Fas and its ligand, Fas ligand (FasL) (31). The molecular mechanism of Fas signaling has been studied extensively (**Fig. 6A**).

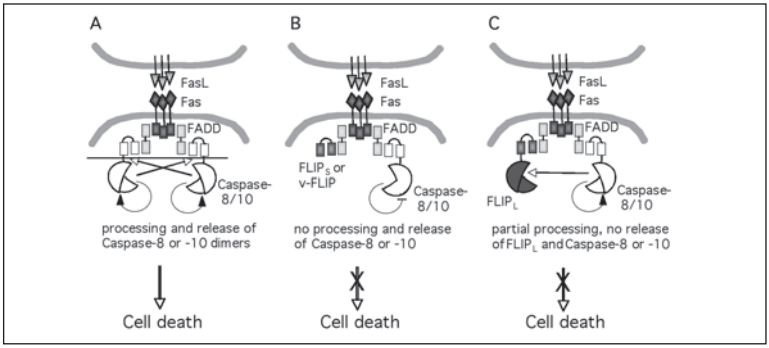

Fig. 6: Role of FLIP proteins in cell death protection of lymphocytes. *Lymphocytes can be triggered to die by membrane-bound FasL, which is expressed on activated lymphocytes. FasL induced trimerization of the death receptor Fas, which assembles a signaling complex that leads to autoprocessing-mediated activation and release of caspase-8 and -10 into the cytosol, to cleave many cellular proteins involved in the execution phase of programmed cell death (**A**). FLIP proteins are structurally homologous to Caspase-8/10, and block Fas-induced cell death by interfering with Caspase-8/10 activation (**B** and **C**). c-FLIPS and v-FLIPs are thought to block Caspase-8 or -10 processing and release, while c-FLIPL allows partial processing of these caspases but no release.*

Fas has a cytoplasmic death domain (DD), and binding of the membrane-bound, trimeric form of FasL to the receptor results in the recruitment of the adapter molecule FADD (Fas-associated protein with death-domain) to the receptor via a DD/DD interaction. FADD also contains an N-terminal death effector domain (DED), which in turn recruits the DED-containing proteases of the caspase family, caspase-8 (formerly called FLICE) and caspase-10 (formerly called FLICE-2), to form a receptor-bound signaling complex (31). It is thought that this process brings two or more caspase-8 (or -10) molecules into close proximity at the receptor level, and allows them to proteolytically activate each other. Active caspase-8/-10 molecules lacking the N-terminal DEDs will then be released into the cytoplasm and initiate apoptosis by subsequent cleavage and activation of downstream effector caspases (caspases-3, -6, -7) and of cellular proteins involved in the execution phase of the cell death program (**Fig. 6A**).

My postdoctoral work has led to the identification and functional characterization of a new class of proteins that prevent Fas receptor-induced cell death (32–34). These cellular caspase-8/FLICE-inhibitory proteins (c-FLIP proteins) are structurally homologous to the Fas associated Caspases-8 and -10, and interfere with their activation by the receptor by preventing their recruitment and/or full processing at the receptor-associated signaling complex (**Fig. 6, B** and **C**).

Two major splice isoforms of cellular FLIP have been described. The long splice isoform, c-FLIP$_L$, contains two DEDs and a protease-like domain, and therefore ressembles Caspase-8 in its overall structure, but lacks critical amino acid residues important for protease activity (**Fig. 6C**). The short isoform, c-FLIP$_S$, lacks the protease-like domain and contains only two N-terminal DEDs that correspond in structure to the FADD-binding N-terminus of Caspase-8 (**Fig. 6B**). FLIPL was found highly expressed in freshly activated T-cells, but is down-regulated upon prolonged stimulation in presence of IL-2, suggesting that decreased FLIP expression levels may sensitize activated T-cells to Fas-induced apoptosis and thus to the termination of the immune response by activation-induced cell death (33, 35, 36). On the other hand, increased expression levels of the short splice isoform of FLIP,

c-FLIP$_S$, has been found in memory T-cell models *in vitro*, suggesting that upregulation of c-FLIP$_S$ could contribute to the increased FasL-resistance of memory T-cells (37, 38). In addition to protecting the lymphocytes from receptor-induced cell death, FLIP proteins can also actively promote lymphocyte activation and proliferation (34). This is most likely mediated by proteins of the TRAF (TNF receptor associated factor) family that bind to c-FLIP$_L$ and certain viral FLIP proteins (39, 40). Viral FLIP (v-FLIP) proteins are present in lymphotropic herpesviruses such as the equine herpesvirus-2 (EHV-2), the herpesvirus saimiri (HVS), but also the human Kaposi sarcoma-associated herpesvirus (human herpesvirus-8, HHV-8 (32, 41, 42). The expression of these v-FLIP proteins is thought to prevent or delay the elimination of herpesvirus-infected cells by cytotoxic T-lymphocytes, by interfering with Fas-receptor-induced cell death signaling (32, 43). Protection against death receptor-induced apoptosis is clearly the best-described function of viral FLIPs, however, additional functions have been discovered. We could recently show that the Kaposi sarcoma-associated v-FLIP protein K13 protects infected cells against superoxide-induced cell death by massive upregulation of manganese superoxide dismutase (MnSOD) (44). The human poxvirus molluscum contagiosum virus (MCV), which infects keratinocytes, contains two quite different v-FLIP variants with unusually long C-terminal extensions. We could show that one of these, the FLIP protein MC159, contains C-terminal binding sites for TRAF2 and TRAF3 that are dispensable for short term protection, but required for efficient long-term protection of cells against Fas-induced apoptosis, an effect that correlated with impaired stimulation-induced internalization of the Fas receptor (40).

Interestingly, we recently found elevated levels of c-FLIP expression in DLBCL cell lines of the ABC subtype, and these were reduced through pretreatment with the MALT1 inhibitor (Hailfinger et al., in press) because the expression of the FLIP proteins themselves is controlled by the transcription factor NF-κB (45, 46). Thus, NF-κB targets such as cellular FLIP proteins may have an important role in the resistance of these particularly aggressive lymphomas against chemotherapeutic or immune response-mediated attempts to stop the disease.

Implications for the design of new immunosuppressive or anti-lymphoma therapies

The study of intracellular signaling pathways that control the proliferation and survival of lymphocytes has led to the identification and characterization of several key components and regulators of these pathways. Proteins that have an enzymatic activity are of particular interest, because of the possibility to develop pharmaceutical inhibitors with therapeutic applications. Calcineurin inhibitors such as cyclosporine A inhibit the antigen receptor-mediated activation of the NFAT pathway and are widely used in immunosuppressive therapies, but have side effects that are due to the ubiquitous expression of NFAT family members in other tissues. New strategies are under development, such as targeting protein kinase C-family members that are specifically expressed in lymphocytes and control the activity of both, the NFAT and NF-κB pathway in lymphocytes (47–49). Therapeutic targeting of signaling components specific to the NF-κB pathway has gained interest because of the discovery of its deregulation in certain types of cancers (26), but also because of its predominant role in controlling many aspects of the immune response (50). The better understanding of NF-κB regulation therefore holds the promise to develop drugs that can either interfere with the pathway to dampen exaggerated immune responses, or to inhibit cellular proliferation in specific subtypes of lymphomas characterized by constitutive NF-κB activation. MALT1 and its binding partners, CARMA1 and BCL10 are key players in this pathway (11, 29), but also affect other T-cell responses such as cytoskeleton-dependent changes in the lymphocyte cell shape (6) and an increased adhesiveness to antigen presenting cells (19). The further elucidation of these and possibly other, yet to be discovered functions of these proteins is a challenge for our future research.

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