UvA-DARE (Digital Academic Repository)

Survival of the fittest clone: Pro-apoptotic protein Noxa controls selection of lymphocytes under competitive conditions
Wensveen, Felix

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
CHAPTER I:

Introduction and Thesis outline

Felix M Wensveen¹

¹Department of Experimental Immunology, Academical Medical Center, Amsterdam, The Netherlands
Chapter I

Prelude

The central concept of this thesis is competition driven selection and survival, or to put it in more popular terms, Darwinistic ‘survival of the fittest’. Darwinistic selection is commonly appreciated as a process that drives the generation of species by allowing survival of organisms in with favorable, specialized characteristics for a certain niche. This enables them to pass on their genes to their progeny, thus acquiring dominance within the population. An important feature of Darwinistic selection is that it is not sufficient for the ‘fit’ organisms to survive (positive selection) in order to obtain dominance within a niche. Selection only gets meaning when the ‘unfit’ organisms are actively eliminated (negatively selected) from the population, thus generating space within the limited niche for the ‘fit’ organisms to thrive and expand.

This thesis does not focus on Darwinistic selection for the generation of species, but for the formation of specialized cell subsets of the hematopoietic lineage within the same organism. Favorable features in this case are not bigger teeth or stronger legs, but more receptors for survival factors and better control of suicide mechanisms. Negative selection is not death of the organism, but cell death mediated by apoptosis.

In order to clarify the chapters of this thesis, the following sections within this introduction will introduce the central topics on which these chapters are built. First the molecular mechanism of apoptosis is introduced. Second, a special focus will be given to the apoptotic molecule Noxa, which takes a special place within this thesis for its important role in selection based survival. Next, the niches of the cellular subsets on which we focus in this thesis, being red blood cells, T cells and B cells, will be introduced: the hematopoietic system and the adaptive immune system. This introduction will be concluded with a general thesis outline.

Apoptosis

All phases in the life of a cell are highly regulated, up to and including cell death. The end of a cell can be executed via several different molecular mechanisms, including necrosis, pyroptosis and autophagic cell death (1-3). However, by far the most well described mechanism of cell death is apoptosis. Apoptosis (Greek for “dropping off”) was already identified in the early seventies (4) as it can be clearly recognized in culture by distinguished morphological features. When a cell undergoes apoptosis, its nuclear material condensates and later the cell starts to generate membrane protrusions, or blebs, which ultimately detach from the cell to be phagocytosed by neighboring cells.

On a molecular level, apoptosis is characterized by the activation of a group of proteases, known as caspases. The caspases cleave their target in a highly regulated fashion at well defined recognition sites (5). Functionally, with the exception of caspase 14, caspases can be subdivided in three different groups: Initiator caspases, effector caspases and inflammatory caspases. Of these, the first two groups are primarily involved in induction of apoptosis, even though other functions have been reported (6). All caspases have in common that they are present in virtually every cell type in an inactive form and require post-translational modification for their activation. Initiator caspases, such as caspase-8 and -9, need to multimerized and undergo proteolytic (self-)cleavage to allow their activation and initiate
apoptosis. Once active, initiator caspases cleave effector caspases such as caspase 3 and 7, thus activating them by exposing their proteolytic domain. The effector caspases initiate activation of a series of DNAses, RNAses and proteases which degrade cellular material in an orderly fashion. In addition, caspases cleave anti-apoptotic proteins, as well as themselves, thus generating self-amplifying and feed-forward loops characteristic of a “point of no return” in cell fate decision.

Since proteolysis is an irreversible process, activation of caspases is very strictly controlled. Inhibition of caspase activity is mediated at various levels, primarily by a group of molecules called the Inhibitors of Apoptosis (IAPs). These proteins are structurally diverse, but all share a so called BIR domain. Well documented is the activity of XIAP, which binds on the proteolytic site of activated caspases, thus blocking their activity. Many cancers have been shown to overexpress XIAP in order to prevent their execution. Other IAP members, such as cIAP1 and cIAP2 are responsible for the ubiquitination and proteasomal degradation of caspases. Finally, several molecules that are not structurally related to IAPs, such as FLIP and ICEBERG, directly bind to caspases and thus prevent their recruitment in an activating complex.

Initiation of caspase activity is mediated by two different signaling pathways: The extracellular and the intracellular cell death pathways. The extracellular cell death pathway is executed by so called “death receptors” such as FAS and the TRAIL receptors. Upon ‘death ligand’ binding, these molecules of the TNF-superfamily allow the formation of intracellular signaling complexes, which may ultimately lead to the activation of caspase-8. FAS, TRAIL and TNF molecules are therefore generally appreciated as inducers of cell death. However, it depends on the presence of the right intracellular components whether these molecules function as death inducers or as mediators of pro-survival signals, as molecules such as TNF and FASL are also reported to stimulate tumor cell survival.

As its name implies, the intracellular cell death pathway mediates responses to apoptotic stimuli that come from within the cell, such as DNA damage, cytotoxic stress and a lack of pro-survival signaling as a result of cytokine deprivation. The intracellular cell death pathway functions by control of mitochondrial outer membrane integrity. The group of molecules that is primarily responsible for this control is the Bcl-2 family of pro- and anti-apoptotic proteins. The Bcl-2 molecules can be divided in three different groups, based on structural homology and function. The pro-survival proteins, including Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1/Bfl-1 share four Bcl-2 homology domains and are primarily responsible for antagonizing the pro-apoptotic BH3-only molecules. This still growing group of molecules includes Bim, Bid, Puma, Noxa, Bik, Harakiri and Bmf and shares only a single Bcl-2 homology domain (BH3) with the other Bcl-2 family members. However, it is this BH3 domain which is primarily responsible for its pro-apoptotic function, as BH3 peptides alone are able to induce cell death in cell culture systems. An important link between the extracellular and intracellular cell death pathways is formed by Bid, as its cleaved and active truncated form (tBid) is generated by caspase-8 activity.

In response to apoptotic stimuli, the activity of the BH3-only group of molecules predominates over that of the pro-survival Bcl-2 like molecules, leading to the activation of the third group of Bcl-2 molecules, i.e. the Bax/Bak-like proteins. These molecules, which have three Bcl-2 homology domains, oligomerize upon activation in the mitochondrial outer membrane.
membrane, thus forming pores large enough for proteins to go through. When this happens, molecules like OMI and AIF are released, which can directly induce protein and DNA degradation. In addition, cytochrome C is released. Since this molecule normally involved in the cell’s respiratory cycle during oxidative phosphorylation, mitochondrial inner membrane potential is rapidly lost, resulting in a failure to generate ATP. But more directly, once cytochrome C is release in the cytoplasm, this molecule hetero-oligomerizes with the proteins Apaf1 and caspase-9, which are both present in the cytoplasm. This protein complex called the apoptosome subsequently cleaves effector caspas, thus initiating cell death.

Clearly, the balance between the three groups of Bcl-2 molecules determines whether a cell lives or dies. This balance has therefore also been called the ‘apoptostat’, since it is the expression level of these molecules which determines the sensitivity of cells to specific apoptotic stimuli. Exactly how these three groups of molecules interact remains topic of intense debate (16-18). What is clear is that there is a hierarchical structure within the BH3-only group of molecules (Figure 1, enlargement). Some BH3-only molecules, like Bim, Puma and (t)Bid, bind with various affinities (14) to all pro-survival molecules and overexpression of these molecules cell lines rapidly leads to cell death. Other molecules, such as Noxa, Bmf, Bik and Harakiri, bind only a few of the Bcl-2 like proteins and overexpression of these molecules does not lead to cell death, but sensitizes cells to apoptotic stimuli (16).

Based on these features, two theories are currently favored within the field (19). One model, the direct activation model, states that the potent death inducers Bim, (t)Bid and Puma function as ‘direct activators’, which directly bind and activate Bax/Bak proteins. Bcl-2 molecules function as a BH3-Only ‘sink’ in this model, which bind direct activators to prevent their activity. Weak death inducers like Noxa are thought of as ‘sensitizers’ involved antagonizing Bcl-2 like molecules, thus generating more free direct activators. The indirect activation model presents Bcl-2 like molecules as a Bax/Bak sink that prevents their activity by binding to them. BH3-only molecules function as Bcl-2 antagonists in this model that compete with Bax and Bak for binding. When numbers of active BH3-molecules are increased, Bax/Bak molecules are released and subsequently oligomerize in the mitochondrial outer membrane. Differences in death inducing potential between BH3-Only molecules are primarily the result of differences in Bcl-2 binding promiscuity and affinity in this model. Despite the use of sophisticated in vitro and in vivo models, no conclusive results have yet been generated that can falsify either model.

Independent of what model one favors, the Bcl-2 family of proteins and their BH3-Only antagonists are thought to mediate responsiveness to specific pro- and anti-apoptotic stimuli (19). For example DNA damage, such as induced by gamma irradiation or mutagenic chemicals, specifically induces the upregulation of the p53 responsive genes Puma and Noxa (20). Activation of lymphocytes via their antigen receptor, induces specific upregulation of the NF-κB targets Bcl-XL and A1/Bfl-1, thus making them more resistant to pro-apoptotic triggers (21). Some Bcl-2 family members, such as Bcl-2 and Bcl-XL, are primarily regulated on a transcriptional level. Measuring the mRNA levels of members of this family, for example by RT-MLPA (22), therefore generates a reasonably reliable, cell-specific ‘death profile’, which could predict the responsiveness of cells to certain triggers. However, other members of this family are almost exclusively regulated on a post-transcriptional level, indicating that mRNA- profiling does not generate an unambiguous picture of a
cell’s apoptostat. A special position concerning post-transcriptional regulation is reserved for the pro-survival molecule Mcl-1, a protein that plays a major role in mediating survival in response to cytokine availability (23). Mcl-1 protein levels are thought to be regulated via degradation and small changes in its half-life therefore lead to major changes in its protein levels (24,25). Under steady state conditions, cytokine signaling leads to activation of PI3K. Upon activation, this protein phosphorylates and activates PKB. Active PKB, in turn, phosphorylates and inhibits GSK3β, a kinase of Mcl-1. During cytokine deprivation, PI3K signaling is inhibited, resulting in phosphorylation of Mcl-1 (23). Mcl-1 is thought to be in complex with the E3 ubiquitin ligase MULE (26), and a de-ubiquiting enzyme USP9X (27). Under steady state conditions, the activity of these molecules is in balance. After phosphorylation, USP9X is released and Mcl-1 is ubiquinated by MULE, resulting in its targeting to the proteasome. Thus Mcl-1 functions as an important rheostat for cytokine
sensitivity, deprivation of which leads to rapid decline of Mcl-1 proteins levels and subsequent cell death. In addition to Mcl-1, also its antagonist Bim is target of regulation by phosphorylation. In response to cytokines, activated ERK phosphorylates Bim (28), thus leading to its degradation. Furthermore, PI3K signaling leads to suppression of FOXO3a, which is a transcription factor for both Bim and Puma (29). Thus, on multiple levels the sensitivity of cells for specific apoptotic stimuli is regulated and survival of cells is guaranteed only under the correct circumstances.

**Noxa**

The pro-apoptotic protein Noxa was first identified in 1990 as a molecule that is highly induced in Jurkat cells (an acute T lymphoblastic Leukemia cell line) after stimulation with PMA (30). The protein was therefore labeled ATL-derived PMA-responsive gene (APR) and its gene PMA-inducible protein 1 (Pmaip1). Ten years later, in a screening for p53 responsive genes, it was rediscovered as pro-apoptotic molecule containing a BH3 domain, thus placing it in the BH3-only group of Bcl-2 proteins (31). It was renamed Noxa, Latin for ‘damage’. One year later, when Noxa deficient mice had been generated, it was reported to be one of the major molecules involved in p53 mediated cell death, together with its family member Puma (20).

The human Noxa protein is a small molecule of approximately 8 kDa with a single BH3 domain. Murine Noxa is approximately twice as big and contains two BH3 domains, but is thought to behave similarly to its human homologue. Upon DNA damage, Noxa is highly induced in a p53 dependent manner. However, since its discovery, many other transcription factors have been shown to be able to induce Noxa, including p73, HIF1α and E2f1 (32-34).

Noxa has been classified as one of the apoptotic sensitizers, or indirect activators since its overexpression does not result in cell death, but makes cells more sensitive to apoptotic stimuli (16,17). The cause for its relatively subtle function is thought to be the result of its restricted binding interactions. Noxa was initially shown to interact with Mcl-1 and to a lesser extent to A1/Bfl-1, but not to other pro-survival Bcl-2 family members (35). Recent biochemical studies, however, also show interactions between Noxa and Bcl-XL (36,37), but it remains to be investigated whether this also occurs under physiological conditions.

A unique feature of Noxa is its ability to target its binding partner Mcl-1 for proteasomal degradation (38). As previously mentioned, Mcl-1 is thought to form a complex with the E3 ligase MULE and the deubiquinating enzyme USP9X (26,27). When other pro-apoptotic BH3-only molecules, such as Bim and Puma, bind to Mcl-1 this molecule is actually stabilized, supposedly due to impaired interaction with MULE (39,40). The BH3-domain of Noxa, on the other hand, in combination with other structural domains of this protein, induces degradation of Mcl-1, possibly by impairing USP9X activity or facilitating Mcl-1 and MULE interactions (38). The consequence of Mcl-1 degradation by Noxa is that treatment of transformed cells with the pharmacological compound ABT-737, which inactivates Bcl-XL, Bcl-2 and Bcl-w (41), makes these cells exceptionally sensitive to Noxa-inducing triggers (42). Dual treatment of cancer patients with ABT-737 and irradiation is therefore a promising new therapy, illustrating the clinical relevance of apoptosis research.

Recently a role for Noxa in the immune system was shown in the very cells in which it was originally identified: activated primary and leukemic T cells (43). Upon PMA
stimulation, but also after T cell receptor stimulation and other proliferation inducing triggers, Noxa was strongly induced. Further analysis revealed a survival advantage for cells in which Noxa was artificially knocked down under conditions of limiting nutrient concentrations (43). It was therefore suggested that Noxa may play a role in the expansion phase of activated T cells, when cells have to compete for antigen, cytokines and nutrients (44). Further in vivo evidence for a role of Noxa was shown in Bmi1 deficient animals, which fail to repress Noxa in activated T cells, thus forming reduced numbers of specific subsets of Th2 CD4+ memory T cells (for further explanation of T cell subsets see below) (45). An in vivo function for Noxa in recently activated T cells, however, has yet to be revealed.

Hematopoietic differentiation

Hematopoiesis, the generation of differentiated cells from pluripotent hematopoietic stem cells (HSCs), is an extensively regulated process which results in the formation of all types of blood cell lineages (Figure 2). An important characteristic of the HSC is its ability to undergo self renewal and the population of HSCs maintains a steady state level throughout life (46). During each division, a decision is made resulting in either self-renewal or differentiation, which can result in either symmetric or asymmetric division. Asymmetric division gives rise to one daughter cell which retains its parental phenotype and another so-called short-term stem cell. The latter retains the ability of self-renewal, yet is much more metabolically active and divides more frequently. This short-lived effector cell gives rise to multipotent progenitors, which lose their ability to self-renew, yet still have the capacity to differentiate into all lineages (47).

Stepwise development of multipotent hematopoietic progenitors into terminally differentiated cells is directed by the interplay of extracellular cues with intracellular signaling networks, ultimately leading to a transcriptional profile which determines cell fate. In addition, control of apoptotic molecules, such as Bcl-2 and Mcl-1, determines whether a cell is allowed to initiate differentiation or undergo apoptosis (48). In order to facilitate differentiation and survival, multipotent progenitors express many receptors for soluble factors that may ultimately initiate lineage commitment upon encounter of their ligand. For example, in response to IL-7, multipotent progenitors can differentiate into a common lymphoid progenitor (CLP), which gives rise to all cells of the lymphoid lineage, including NK cells, B cells and T cells (49). Expression of the IL-7 receptor α chain is therefore an important characteristic of CLPs and their progeny. The myeloid lineage is characterized by the absence of IL-7Rα and instead myeloid precursors express the receptor for IL-3. This lineage ultimately gives rise to granulocytes, monocytes, megakaryocytes and red blood cells (50) (See Figure 2).

Proteins of the Bcl-2 family play an important role in mediating hematopoietic cell survival. HSCs express high levels of the pro-survival molecule Bcl-2, which is lost upon differentiation (48). When committed lymphoid progenitors are taken out of their niche, not only do they fail to differentiate into committed lineages, but they also undergo apoptosis due to a lack of survival factors (51). This shows that lineage committed cells highly depend on
Chapter I

Figure 2. Hematopoietic lineage diversification. Long term (LT) hematopoietic stem cells (HSCs) have the ability for lifelong self-renewal and multilineage differentiation potential. LT-HSCs give rise to short term (ST) HSCs, which retain the ability for multilineage differentiation, but have decreased self-renewal potential. According to the generally accepted model, ST-HSCs produce multi-potential progenitors (MPPs), which have completely lost self-renewal potential, but are able to generate all hematopoietic cell lineages. Common lymphoid progenitors (CLPs) are thought to give rise to T, B and NK cells, whereas the common myeloid progenitors (CMPs) give rise to granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs). BFU: Burst forming unit. CFU: Colony forming unit. Adapted from Dr. C. Geest (with permission).
extracellular stimuli, such as cytokines, in order to maintain balance between pro- and anti-apoptotic Bcl-2 family members. For example, CLPs depend on IL-7 signaling in order to downregulate Bim by the MAP kinase ERK (52). IL-3 signaling, a hallmark of the myeloid lineage is required to maintain levels of the pro-survival molecule Mcl-1 (23,51).

Many of these hematopoietic cytokines are exclusively present in niches in the medulla of the bone marrow and this organ is therefore the primary site of haematopoiesis, both in humans and in mice. A notable exception is murine stress erythropoiesis. Under homeostatic conditions, red blood cells are produced by the bone marrow and only a small amount of extra-medullary erythropoiesis can be observed in the spleens of these animals (53). However, during acute anemia, in response to increased levels of the cytokine erythropoietin (epo), extra-medullary erythropoiesis is vastly increased, whereas bone marrow output remains unaffected (54). This indicates that during homeostasis, epo concentrations limit differentiation and/or survival of red blood cell progenitors outside of the bone marrow, thus setting a maximum to the amount of cells that these extra-medullary sites can support. It remains to be investigated whether epo is the only determining factor for erythroid cell numbers in extra-medullary niches, or that other factors such as SCF, which has been shown to be essential for red blood cell development (55), also play a role.

The importance of the Bcl-2 family members in the control of apoptosis during erythropoiesis has been well established. Bcl-XL mediated control of Bax is essential for maturation of erythroid progenitors during erythropoiesis. Conditional Bcl-XL deficient animals in the erythroid lineage rapidly die as a result of severe anemia (56). In addition, Epo-mediated inhibition of Bim via activation of the MAP kinase ERK also plays a major role in the survival of early erythroid precursors (57). Bim deficient mice, however, do not suffer from severe abnormalities in the erythroid compartment. Therefore it is likely that other BH3-only molecules such as Noxa play a role during homeostasis to counter pro-survival Bcl-2 molecules in red blood cell progenitors and prevent erythrocytemia.

In summary, hematopoietic differentiation is an extensively regulated process which highly depends on a proper control of apoptotic molecules to allow proliferation and differentiation. Cytokine mediated control of cell death may therefore be an important regulatory mechanism to determine the population size of differentiated cells, especially in extra-medullary sites.

### The immune system

The immune system is an organ with many different, highly specialized cells with the remarkable feature that they are not restricted to a single location, but are divided throughout the body as single cells or congregated in small clusters. All cells, however, share the same overall goal: to protect the body against invading pathogens and tumors. The immune system can be subdivided in two major arms; the innate and the adaptive immune system. Both arms have separate function, yet extensively interact.

As its name implies, the innate immune system is completely functional at the birth of an organism. Its aim is to control infection and to recruit more specialized cells if its response is insufficient to clear the imposing danger. The innate immune system consists of many different cells, all with highly specialized functions, each involved in warding off different dangers. Recognition of pathogens primarily occurs by binding of a specific set of receptors to
common pathogen associated molecular patterns (PAMPs). The major difference between the innate and adaptive immune responses is that the former is incapable of learning and will approach each pathogen as if it is encountered for the first time. Therefore it is less well equipped to target pathogens or tumors that have acquired features that shield or hide their PAMPs to evade recognition.

The adaptive immune response consists of Bone Marrow derived and Thymus derived cells, or B and T lymphocytes for short. B cells are responsible for the elimination of soluble pathogens and toxins (antigens) via the production of soluble receptors called antibodies. T cells are involved in the elimination of infected cells, which they recognize via interactions of their antigen receptor with non-self peptides presented in MHC molecules on infected cells. Multiple subspecialized subsets can be distinguished within the T cell compartment, but the major distinction can be made based on their expression of CD4 or CD8. These markers correspond reasonably well with their functional differences. CD8 positive T cells are involved in the recognition and subsequent killing of infected cells via a range of cytotoxic molecules. CD4 positive T cells primarily modulate immune responses via direct cell-cell contact with molecules like CD40L, or by the secretion of cytokines. Rather than recognizing a limited set of PAMPs, B and T cells recognize a vast array of different molecules via their B or T cell receptor (BCR or TCR) respectively. This is possible because these receptors are not the product of a single gene, but consist of a patchwork of gene elements that are combined via homologous recombination to form a protein of similar, yet never identical structure in every T and B cell (Figure 3). Additional diversity is obtained by the random addition and/or removal of nucleotides at the sites of recombination, which finally results in a receptor repertoire with a potential diversity of approximately $10^{18}$ combinations (58).

A unique feature of the adaptive immune system is that it is able to ‘learn’. Because of its huge diversity, before antigen-encounter, every clone is in principle only present in a single copy. Many of these copies will never encounter their antigen and therefore they will always remain in a ‘naïve’ state. Upon recognition of its ligand, a lymphocyte is activated in a tightly controlled mechanism, which will be described in more detail below. Upon activation, lymphocytes rapidly proliferate to form millions of clones to battle ‘their’ pathogen. After the pathogen is cleared, most of these cells die via apoptosis, but a small fraction remains as memory cells. These cells are not only present in higher frequency than naïve clones, but also respond much more rapidly and effectively than naïve cells upon antigen-recognition, thus providing the organism with long lasting immunological memory.

**Lymphocyte selection and survival**

T and B lymphocytes differ from other leukocytes by a number of unique features: (1) they have a pathogen-recognition receptor which is unique for every individual cell, (2) they have an unequalled potency to proliferate and (3) they have the ability to differentiate from a naïve ‘unlicensed’ state to a hyper-responsive memory state. In addition to the obvious benefits that lymphocytes provide because of these features, T and B cells also form a high safety risk for an organism: a lymphocyte that recognizes proteins from the own body can cause devastating auto-immunity, uncontrolled proliferation leads to aggressive leukemia’s and improper memory leads to unlicensed activation of a highly cytotoxic subset of cells.
Therefore, the life cycle of a lymphocyte is tightly controlled by a number of stringent selection mechanisms, in most of which apoptosis plays a prominent role.

The first selection phase in the life of a lymphocyte takes place during the formation of its antigen receptor. Because antigen receptor formation is a random process and involves the addition and or removal of nucleotides, antigen-receptor formation often results in an out-of-frame shift. Since recombination can occur on two alleles, an out of frame shift can be rescued by a correct rearrangement on the second allele, but still results in the elimination many cells (59,60). Both T and B cell receptors consist of two rearranged elements. BCRs consist of a heavy and a light chain, TCRs of an alpha and beta chain or a gamma and delta chain. Selection after rearrangement therefore occurs in two phases, intersected by a period of proliferation. Correct rearrangement of a (pre) B or T cell receptor leads to survival signals into the cell (61). Accordingly, mice overexpressing Bcl-2 have increased numbers of cells with nonsense-rearrangements (62). It is important to note that during this phase, T cells are

---

**Figure 3. T cell receptor re-arrangement.** The T cell receptor complex consists of a variable α and β chain, flanked on the membrane by the constant γ, δ and ε chains and intracellularly by the ζ chain. The variable α and β chains are generated by recombination of structural gene element to form two basically unique proteins. During segment rearrangement, random basepairs are added and removed at the sites of ligation to increase receptor diversity. The α chain consists of a Variable (V), Joining (J) and Constant (C) region. The β chain also has a Diversity (D) region, which further increases potential diversity. For a color figure, see Appendix III.
selected for the recognition of MHC molecules (63). B cells, on the other hand, depend on ‘tonic’ BCR signaling to remain viable (64).

In the second selection phase of lymphocyte biology, cells are negatively selected for self recognition. Also during this phase the antigen receptor plays an important role, but now to transfer pro-apoptotic signals into the cell. The dual role of the antigen receptor in mediating pro- and anti-apoptotic stimuli depends on the signal strength after ligand binding. No signal results in reduced A1 upregulation and a failure to inhibit FOXO3a and p53, which induces apoptosis via induction of Bim and Bid (65,66). Persistent or (too) much signaling strength on the other hand, results in upregulation of Bim and FAS (67,68). Mice lacking Bim, or the natural mutants lpr/lpr and gld/gld, which lack FAS or its receptor respectively, have greatly impaired negative selection, resulting in auto-immunity at a later age (69-71).

In addition to antigen receptor signaling, the cytokine environment is essential for survival of lymphocytes during their development. For B cells it has been shown that BAFF and IL-7 play a crucial role (52,72). Not only are these factors involved in lymphoid differentiation, but they also inhibit induction of Bim via the ERK and PI3K pathways respectively. IL-7 receptor knock-out mice almost completely lack B cells, whereas when this mouse also lacks Bim this phenotype is largely reversed (52). T cells also depend on IL-7 for their survival, even though less so than B cells in mice (73). Interestingly, in humans this phenotype is reversed, as people lacking the IL-7 receptor alpha chain are almost devoid of T cells, whereas their B cell compartment is relatively normal (74). A special role in lymphocyte biology is reserved for Mcl-1. As previously mentioned this molecule is highly responsive to cytokine availability (23) and was shown to be essential for lymphocyte survival (75). Mice conditionally lacking Mcl-1 in the T cell compartment rapidly lose their T cells already during the double-positive thymocyte stage. Cells which conditionally delete Mcl-1 in B cells die as soon as this molecule is lost (75).

After they leave their sites of origin, lymphocytes continue to require pro-survival signals in order to persist in the periphery. Of major importance for peripheral lymphocyte survival is the antigen receptor. B cells that are induced to lose their BCR die within days (76). Recently it was discovered that signaling mediated by PI3K plays an important role in mediating these effects (64), which suggests an important role for Mcl-1. Ligands responsible for this ‘tonic’ BCR signaling remain yet to be discovered. Naive TCR-less T cells also die, yet not as rapidly as BCR-less B cells (77). Tonic signaling via MHC molecules seems to play an important role in the maintenance of naive T cells, as cells transferred to MHC-deficient mice have a much higher turnover than cells transferred to WT animals (78).

Activation of naive T lymphocytes results in a phase of rapid proliferation and subsequent differentiation into highly cytotoxic cells specialized in the elimination of infected cells or the production of inflammatory cytokines. Activation and survival of these cells is therefore very tightly regulated and subject to at least three layers of control. The primary license for lymphocyte activation and survival is recognition of antigen by its unique antigen receptor. Ligation of this molecule leads to activation of the NF-kB, MAPK and PI3K signaling pathways and induction of molecules like A1 (79). However, if naive cells only receives activating signals from its antigen receptor, expression of these molecules is not maintained and instead the expression of FAS is induced, resulting in a process called
activation induced cell death (80). Cell death is prevented, however, if a second survival signal is given in the form of cell surface (co-stimulatory) molecules.

Expression of co-stimulatory molecules is restricted to specific cell subsets of the immune system, generally referred to as professional antigen presenting cells (APC). One of the most prominent APC’s is the dendritic cell, which is responsible for the transport of antigen from the periphery to the lymph nodes. If this cell is activated via its PAMPs, it does not only present antigen, but also the required co-stimulatory molecules on its surface. For B cells, this so called second signal is primarily given by CD40L (80). Binding to this molecule maintains NF-kB expression and thus persistence of Bcl-XL, A1 and also Mcl-1 (81). T cells depend on interactions between members of the CTLA4 family, most importantly CD28 and its ligands B7-1 and -2, or members of the TNF-superfamily, such as CD27 and its ligand CD70 (82,83). CD28 ligation leads to the induction of PI3K and also to the upregulation of nutrient receptors (84). CD27 ligation results in increased NF-kB signaling and also in enhanced third signal induction: production of cytokines (85).

Cytokines form a functionally and structurally diverse group of excreted molecules that appear to modify the immune response, rather than being individually necessary. B cells, for example, require IL-4 for switching to the IgG1 subclass (86). However, animals deficient for these molecules still produce significant amounts of IgG1 upon infection. Other molecules, such as BAFF, appear to be more essential for the survival of activated B cells, as conditional BAFF knock-outs fail to support the survival of germinal center B cells (72). Activated T cells rapidly upregulate receptors for the molecules IL-2 and IL-15 (87). However, mice deficient for these molecules still have largely normal T cell responses, dependent on the pathogen they encounter (88-90). Unclear is whether this is the result of redundancy in the system, or that these molecules have evolved to function only in specific immune responses. Also, it remains to be investigated what the influence of limiting cytokine concentration is on the outgrowth of specific immune populations.

The next selection phase in the life of a lymphocyte is the period after expansion has reached its peaked and the pathogen is cleared. As most antigen-specific cells become superfluous, the majority of cells die via apoptosis and only a small minority remains as memory cells. It is still a matter of intense debate whether memory cells arise early in the immune response, during a decision moment that differentiates part of the activated cells into a memory fate and the rest in a short-lived effector fate, or that memory cells arise later on, as survivors during the contraction phase (91,92). What is clear is that the absence of cytokines and antigen quickly induces apoptosis. Several papers, combining absence of Bim with deficiency for FAS in mice have shown that it is primarily the intracellular cell death pathway that is responsible for this contraction, illustrating the importance of cytokines and the Bcl-2 molecules for (activated) lymphocyte survival (69-71). The cells that survive the contraction phase have obtained two basic requirements: (1) they have receptors for survival factors like IL-7 that are produced (also) in the absence of inflammation and (2) they have reached niches in the body where these factors are present. The most illustrative example form long-lived plasmacells. These terminally differentiated cells acquire receptors for IL-6 which ensures their survival (93). However, only if they reach a restricted number of niches in the bone marrow, where sufficient amounts of IL-6 are present are these cells allowed to survive for
long periods of time (94). For memory T and B cells these requirements are less restricted, even though they still require pro-survival signals in order to be maintained (95,96).

The final selection phase for lymphocytes is reactivation of memory cells after re-encounter of antigen. As previously indicated, memory cells have already proven themselves to be specific and effective and their expansion is therefore much less restricted by control mechanisms. Even so, it has been shown that re-encounter of pathogens further narrows the diversity of the antigen-specific repertoire, suggesting that selective mechanisms do take place (97,98). It remains to be investigated, however, if apoptosis plays a role during this phase of lymphocyte biology.

**Thesis outline**

Selection is based on intrinsic capacity, relative to that of its competitors in a given niche. In immunology, apoptosis has been well established as the main executor of cellular selection. In this thesis we set out to investigate in vivo how members of the Bcl-2 family of pro- and anti-apoptotic proteins contribute to the formation of differentiated cell populations from the hematopoietic lineage when these cells have to compete with each other for growth factors and nutrients based on their intrinsic features. A special focus will be on the role of the pro-apoptotic protein Noxa in these processes, in interaction with Mcl-1 and Bim.

Using in vitro systems, more insight will be gained in the biochemical mechanisms that modulate the Noxa/Mcl-1 axis under competitive conditions of limiting nutrient availability (chapter 2). A combination of human and murine models will shed more light on how these mechanisms influence cytokine-controlled erythropoiesis (chapter 3). Infection and immunization models in vivo investigate the role of apoptosis in the generation of effector and memory T and B cell populations (chapters 4-6). Chapter 4 focuses on the role of Noxa and Mcl-1 in the formation of high-affinity effector CD8⁺ T cells. Chapter 5 expands on this study and describes how Noxa influences memory CD8⁺ T cell formation. In Chapter 6 the role of Noxa in the generation of high-affinity B cell responses is investigated. Finally, we study how co-stimulation via CD27 influences the balance between apoptotic signaling cascades (chapter 7) and how this molecule affects effector and memory T cell formation.

**Reference List**


