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The immunomodulatory properties of C-Med 100® an extract of the plant *Uncaria tomentosa*

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Abstract

Extracts from the bark of the vine Uncaria tomentosa have anti-inflammatory, antiviral, antimutagenic and antioxidant effects. In this study, we evaluate the effects of an Uncaria tomentosa extract, denoted C-Med 100, on the cellular level of the immune system both in vivo and in vitro. Our results indicated that lymphocytes had significantly prolonged half life after in vivo supplementation with C-Med 100. This phenomenon significantly increased the number of all leukocyte subsets in the spleen without influencing proliferation capability or cell cycle progression after in vitro stimulation. Fractionation of the extract identified quinic acid (QA) as a possible active component and supplementation with QA significantly increased the number of spleen cells, recapitulating the in vivo effect of C-Med 100. Furthermore the in vitro studies revealed that NF-kB activity was significantly inhibited in both C-Med 100 and QA treated cells, providing an explanation for the reported anti-inflammatory effects of Uncaria tomentosa extracts. C-Med 100 also inhibited the proliferation of normal mouse T and B lymphocytes as well as various tumour cell lines. The inhibition was not caused by toxicity or by induction of apoptosis and no discrete cell cycle block was observed. In contrast, QA did not interfere with cell proliferation, suggesting that inhibition of proliferation and NF-kB activity were independent events.
Collectively our study has identified QA as a candidate constituent of the C-Med 100 extract inducing increased cell numbers in vivo and inhibiting NF-kB activity in vitro.

Key words: Uncaria tomentosa, quinic acid, NF-kB, inhibition, proliferation, survival

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To my family

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Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-III).

I. Åkesson Ch., Pero R.W. and Ivars F. 2003. C-Med 100®, a hot water extract of *Uncaria tomentosa* prolongs lymphocyte survival *in vivo*. *Phytomedicine* 10: 23-33

II. Åkesson C., Lindgren H., Pero R.W., Leanderson T. and Ivars F. 2003. An extract of *Uncaria tomentosa* inhibiting cell division and NF-κB activity without inducing cell death. *Int Immunopharmacology* 3: 1889-1900

III. Åkesson C., Lindgren H., Pero R.W., Leanderson T. and Ivars F. 2004. Quinic acid is a biologically active component of the *Uncaria tomentosa* extract C-Med 100®. *Manuscript*

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Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immuno-deficiency syndrome
Ap-1	activator protein-1
APC	antigen presenting cell
BAFF	B-cell activating factor
BCR	B cell receptor
BrdU	5-bromo-2-deoxy-uridine
CAE	carboxy alkyl ester
CAPE	caffeic acid phenethyl ester
CD	cluster of differentiation
CFS	chronic fatigue syndrome
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CTL	cytotoxic T cell
DAG	diacylglycerol
DC	dendritic cell
GALT	gut-associated lymphoid tissue
GI	gastrointestinal tract
G-CSF	granulocyte colony-stimulating factor
HTLV	human T-lymphotropic virus
IAP	inhibitor of apoptosis protein
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of κB
IKK	IκB kinase
IL	interleukin
IP_3	inositoltriphosphate
ITAM	immunoreceptor tyrosine based activation motif

LFA	leukocyte functional antigen
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MEKK	MEK kinase
MHC	major histocompatibility complex
NF-AT	nuclear factor of activated T-cells
NF-κB	nuclear factor κB
NIK	NF-κB inducing kinase
NK	natural killer cell
NKT	natural killer T cell
NSAID	non-steroidal anti-inflammatory drug
PALS	periarteriolar lymphoid sheath
PBMC	peripheral blood mononuclear cell
PIP_2	phosphatidylinosotol diphosphate
PLC	phospholipase C
QA	quinic acid
QAA	qunic acid ammonia salt
QAL	quinic acid lactone
Rf	retention factor
SMAC	supramolecular activation cluster
TCR	T cell receptor
TD	thymus-dependent
TGF	tumor growth factor
Th	T helper
TI	thymus-independent
TLC	thin layer chromatography
TNF	tumor necrosis factor
TNF-R	TNF receptor
US	United States of America

General introduction

The immune system is commonly divided into two parts, the innate and the adaptive immune systems. The innate immune system is represented by physiological barriers such as skin and mucosal surfaces together with phagocytic cells, granulocytes and natural killer (NK) cells and soluble components such as defence proteins which have the ability to mark and destroy pathogens. The components of the innate immune system are always present and strikes directly by producing a response against the foreign invaders.

Cells of the adaptive immune system are resting and in response to infection they divide and differentiate to acquire the effector function. Thus it takes some time to generate an immune response to infection. The induction of a highly specific response involves antigen specific T cells that either kills infected cells or helps B cells to become activated and the activated B cells in turn secrete specific antibodies directed towards the microorganism. Finally, during the adaptive immune response memory cells are generated. Memory cells remain in the organism for a long time and can directly mobilize a specific and rapid response if or when the same infection tries to invade the host again. The memory cells and the specific antibodies that are generated during an adaptive response explain why we only become infected once in our life by for example rubella and also why vaccinations can protect us. We may become infected repeatedly but disease will not develop because the memory cells will stop the invasion before we notice it is there.

Pharmacologically active products often help us feel better during the time the adaptive immune response develops, which is the period when the immune system is mounting an attack against the invading pathogen. Furthermore various diseases can be induced by a defective immune system in itself; i.e. autoimmune diseases

and allergy. In addition, AIDS and cancer are other complex diseases that often need pharmaceutical treatments and there is a continuous search for new more potent substances to fight such disorders. Currently, numerous components from plants are known to possess qualities that might be beneficial to treat disease. However many of these components have not yet been well characterized and more studies are needed for successful pharmaceutical development. Mankind began to produce remedies from nature, against various disorders, thousands of years ago and today twenty-five percent of our medical drugs originate from natural products and new useful components are still discovered.

The focus of this thesis will be on how an extract from the vine *Uncaria tomentosa* influence the cellular mechanisms of the immune system. I more specifically investigate a water derived extract from *Uncaria tomentosa* called C-Med 100® which presently is sold as a dietary supplement in the US. This plant grows in the Peruvian rainforest and has been used by the Asháninka Indians for treating various diseases. Studies have been performed to characterize the biological mechanisms that mediate its efficacy, and what the active component(s) of the plant extract might be. In this thesis we wanted to continue these investigations and focus on how C-Med 100® modulates different cellular mechanisms in the immune system.

Our findings on how C-Med 100® affects immunity are presented in paper I-III. In addition, I have summarized how compounds in plants are metabolized in organisms and how these metabolites might influence immunity. The relationship between metabolism of compounds and their mode of action in the body are closely connected and metabolism can interfere with the outcome of pharmaceutical treatments. Furthermore the immune system and important cellular mechanisms that control immune responses are described.
The most important finding of this thesis was the identification of quinic acid as a biologically active component of the C-Med 100® extract. We showed that this

component prolongs leukocyte life time *in vivo* and inhibits the activity of transcription factor NF-κB, which is central in initiating inflammatory responses. In addition, we show that C-Med 100® treatment inhibits cell growth without inducing cell death *in vitro*. This type of mechanism for an anti-inflammatory component appears to be quite rare and may prove to be useful in the future as a clinical treatment of inflammatory diseases.

Central lymphoid organs

Bone marrow

The embryonic development of blood cells (hematopoiesis) occurs in the yolk sac, in the mesenchyme around the main arteries, in the fetal liver, the bone marrow, the thymus and more temporarily in the spleen (reviewed in [1]). After birth and in adult life hematopoiesis takes place in the red bone marrow inside our skeletal bones. At birth the bone marrow primarily is hematopoietic but later most of the red bone marrow is replaced by yellow bone marrow and in humans there is approximately a ten percent decrease in cellularity of the red bone marrow for each decade of life (reviewed in [2]). In adults red bone marrow exist in the proximal epiphyses of the humerus and femur, in the vertebrae and in flat bones such as sternum, ribs, cranical bones and pelvis.

In the red bone marrow, pluripotent hematopoietic stem cells (pHSC) are throughout life responsible for the production of all subsets of blood cells including mature members of the innate or adaptive immune system. There are two qualities that stem cells have to possess to fulfil the demands for the stem cell definition. First, they have to be self-renewable and secondly capable of multilinage differentiation. Furthermore dividing stem cells have four developmental choices: they can be self-renewable, differentiate, enter programmed cell death or emigrate, since the movement of stem cells between various hematopoietic sites occurs naturally. To produce all subsets of blood cells, stem cells first give rise to multipotent progenitors (MPPs) which in turn give rise to either common lymphocyte progenitor (CLP) cells or common myeloid progenitor (CMP) cells. The CLP cells are restricted to develop into T lymphocytes, B lymphocytes or natural killer (NK) cells while the CMP cells are progenitors for the myeloerythroid lineages e.g. monocytes, granulocytes, erythrocytes and platelets. The developing subpopulations of lymphoid or myeloid progenitors differentiate into mature cells by following special differentiation pathways guided by growth and cytokine

receptor signalling together with the activity of transcription factors (reviewed in [3-5]). Today there is much effort in trying to define the different developmental stages of immature lymphocytes. To accomplish this, various surface marker molecules are used to separate the developing cells into early progenitors, late progenitors or immature cells ready to leave the bone marrow and migrate to maintain the pool of white blood cells in the periphery.

The highly regulated process of B lymphocyte development also takes place in the bone marrow. The production of B lymphocytes starts before birth and the process continues throughout life in the adult bone marrow. The commitment into the B cell lineage and the selection of B cells is defined and accomplished by the surface immunoglobulin molecule IgM which is the hallmark of immature B cells. The IgM expressing immature B cells migrate from the bone marrow to the spleen were they complete their maturation.

The membrane bound immunoglobulin (Ig) molecule on the B cell surface serves as the antigen recognizing receptor and is an important part of the B-cell receptor (BCR) complex. B cells produce Ig molecules with numerous antigen specificities and terminally differentiated B cells, called plasma cells, secrete immunoglobulin (antibody). This secreted form of immunoglobulin is the main effector molecule that binds to pathogens or their toxins in the extracellular spaces of the body and the production of these are the most important effector function for B cells.

The peripheral B cell pool can be divided into B1 cells that reside in compartments such as the peritoneal and pleural cavities and B2 B cells which either recirculate through the secondary lymphoid organs or stay as follicular (FO) B cells in the secondary lymphoid organs. Among these two populations the B2 cells are the conventional B cells and the B1 cells are distinguished from conventional B2 cells since they have a self-renewable capability and express special cell surface molecules such as CD5, CD45 and CD43. Furthermore a population called marginal zone (MZ) B cells exist in the spleen. The MZ B cells share many phenotypic characteristics of B1 B cells but they do not express CD5. The

developmental process of these different B cell subpopulations is still not fully understood but one of the most important mechanisms that influence B cell development is the expression of different transcriptional regulators (reviewed in [6-10]).

Thymus

The thymus is a primary lymphoid organ where T cells develop and it is situated above the heart. It is surrounded by a capsule followed by the subcapsular region (SCR), the cortex, the cortico-medullary junction (CMJ), the medulla and these different subcompartments together construct a 3D-network containing various cell types (reviewed in [11, 12]). The size of the thymus reaches maximum shortly after birth and the organ declines with age even though the production of T lymphocytes continues throughout adulthood. The earliest precursors that enter the thymus are the CLP cells that migrate from the fetal liver to the fetal thymus before birth or from the bone marrow to the thymus in adult individuals. Inside the thymus the immature thymocytes move between the different thymic subcompartments in order to become educated and selected for the ability to distinguish between self and non-self antigens. Today these progenitor T cells within the thymus can be divided into different maturation stages with the help of cell surface molecules (reviewed in [13-15]). Furthermore the thymocytes that are selected, during what often is referred to as positive and negative selection, can develop into different functional subpopulations of T cells thereby keeping the different T cell compartments in an organism at a desirable level.

The T cell pool in the peripheral lymphoid organs mainly consists of conventional αβ T cells, which have a TCR consisting of α and β chain proteins. These conventional αβ T cells are further divided into $CD4^+$ helper T cells (Th) or $CD8^+$ cytotoxic T cells (CTL). The Th cells are cytokine producing cells that helps the CTLs in a cytolytic response or the B cells in a humoral response depending on what infectious agents that have triggered the immune system. Activated CTLs are

cytotoxic cells that specifically kill for example infected target cells. Both $CD4^+$ and $CD8^+$ T cells circulate through the lymphoid organs and tissue via the lymphatics and the blood (reviewed in [16-19]). In addition regulatory $CD4^+CD25^+$ T cells are found in the periphery. These cells are considered as naturally suppressor cells since they do not respond conventionally to antigen but instead they suppress the immune response of other T cells [20, 21]. It has recently been suggested that even $CD4^+CD25^+$ regulatory T cells can develop in the thymus [22].
The peripheral T cell pool also consists of γδ T cells which carry TCRs consisting of γ and δ chain-proteins. These cells reside in epithelial tissues and peripheral lymphoid tissues and their function is suggested to be dependent on their tissue distribution. Furthermore it is believed that a part of the γδ T cell population might be extrathymically derived (reviewed in [23, 24]). In the peripheral lymphoid organs a small population of T cells called NKT cells also exist. These cells express intermediate TCR levels and membrane markers in common with NK cells e.g. NK1.1, IL-2β and DX5. The NKT cell population can further be divided into three main categories based upon the expression of certain cell surface molecules and TCR specificity (reviewed in [25, 26]).
These are the main T cell populations that are provided by the thymus in order to keep the peripheral T cell pool at a steady state. All together the process of T cell development is highly regulated and the developmental regulation involves (i) intracellular signalling proteins and nuclear transcription factors (ii) extra cellular signals like cytokines, growth factors and (iii) intercellular communication.

Peripheral lymphoid organs

After development and maturation in the bone marrow and thymus, lymphocytes migrate to the peripheral lymphoid organs which include lymph nodes, spleen and the mucosal lymphoid tissues such as the gut-associated lymphoid tissue (GALT).

In these secondary lymphoid tissues, antigens accumulate and are presented to the different subpopulations of lymphocytes. The rest of the body is considered to be tertiary lymphoid tissues which normally contain small amounts of lymphocytes, but in response to inflammation high numbers of lymphocytes are recruited into these sites (reviewed in [27]).

Lymph nodes

In the lymph node, antigens are filtered out from the lymphoid fluid to become presented on APCs and give rise to adaptive immune responses. The lymph nodes can be divided into two main regions, the cortex and the medulla. The cortex is further divided into the paracortex that consists of distinct T- and B- cell areas. In the paracortex, T cells interact with dendritic cells (DCs) in the T cell area, while the B cell area consists of primary follicles and after antigen challenge, germinal centers. The medulla contains lymph draining sinuses were plasma cells, macrophages and memory T cells are situated. Circulating lymphocytes enter the lymph nodes either via the high endothelial venules (HEVs) or through the afferent lymph in the subcapsular sinus (reviewed in [28]).

Spleen

The spleen filters out antigens from the blood and can be divided into the red pulp which is a site for red blood cell destruction and the white pulp which comprises the lymphoid compartments. The red pulp composes approximately 75 percent of the spleen and it consists mainly of splenic cords and thin-walled sinuses which are primary vascular structures with unique capabilities. This part of the spleen plays an important role in the removal of encapsulated bacteria since the slow blood flow makes it possible for the stationary macrophages in the tissue to phagocytose antigens that are passing trough. Furthermore, tumour cells, red blood cells and platelets are taken up in the red pulp by stationary macrophages. The white pulp composes approximately 25 percent of the splenic volume and consists of T cell

rich periarteriolar lymphoid sheaths (PALS) and B cell rich primary lymphoid follicles. After antigen challenge germinal centers where B cells undergo intense proliferation during an adaptive immune response are generated in the primary lymphoid follicles. Therefore the morphology of the white pulp varies based on the amount of challenging antigens present and the age of an individual (reviewed in [29]).

The blood flows from a trabecular artery, into the central arteriole and further into the marginal sinus that is surrounded by the marginal zone and here the antigens are exposed to the lymphocytes. The lymphocytes that arrive with the blood, migrate out in the PALS were they can interact with antigens from the red pulp. If antigens are not encountered, the lymphocytes migrate out from the white pulp into the red pulp and join the circulation that leaves the spleen out into the body. Furthermore the spleen can enlarge after various treatments, for example after treatment with G-CSF *in vivo* [30, 31]. Thus the weight of the spleen varies and the size of the organ decreases with age (reviewed in [29]).

Gut associated lymphoid tissue (GALT)

The gut associated lymphoid tissue consists of the Peyer's patches, the mesenteric lymph nodes (MLNs) and isolated lymphoid follicles in the gut lamina propria. These are the organized lymphoid tissues of the gut. Included in the GALT are also lymphocytes spread throughout the lamina propria (the lamina propria lymphocytes) and the intestinal epithelium (the intraepithelial lymphocytes). These are two diverse populations of lymphocytes with a memory or previously activated phenotype. The lymphocytes within the GALT have to distinguish between harmful antigens from invading microorganisms and beneficial antigens from food proteins and commensal bacteria, such that tolerance is induced to beneficial antigens and immunity is induced to harmful antigens. However, the exact mechanisms for how tolerance rather than immunity is induced to beneficial antigens is still largely unknown (reviewed in [32-34]).

Lymphocyte circulation

Antigens are transported to the secondary lymphoid organs in various ways; (i) the lymphatic vessels carry antigens to the lymph nodes which are strategically placed through out the body (ii) the spleen filters out antigens that are blood born and (iii) specialized cells in the gastrointestinal tract transport antigens into the Peyer's patches. Antigens can also be carried to the lymph nodes by DCs which capture antigens in peripheral tissues and migrate through the lymph to the lymph nodes. Furthermore the secondary lymphoid organs have a highly specialized system for recruiting lymphocytes from the primary lymphoid organs and the blood. This recruitment is not random but rather it is regulated by active mechanisms such as lymphocyte-endothelial cell recognition [35] and most mature lymphocytes circulate continuously between the blood and the secondary lymphoid organs. The lymphocyte circulation is highly efficient and individual cells inspect several secondary lymphoid organs for the presence of their specific antigen in a matter of days. Interestingly, one lymph node is said to recruit two percent of the entire recirculating lymphocyte pool in a day [36]. The immune system manages to regulate the migration of naïve, effector and memory lymphocyte populations and to distribute them to different body compartments. In general, naïve lymphocytes "home" to the secondary lymphoid organs and continue to migrate between these organs and the blood until they either die or encounter antigen. The homing behaviour of naïve lymphocytes is homogenous even though the T lymphocytes home to the T cell area and the B lymphocytes home to the B cell area in the secondary lymphoid organs. Furthermore the memory and effector lymphocyte subpopulations mainly access tertiary tissues but parts of these subpopulations traffic through secondary lymphoid organs [37, 38]. Thus the homing pattern for memory and effector cells is often heterogeneous and tissue selective (reviewed in [28, 39, 40]).

Today the overall view is that lymphocyte homing is orchestrated and controlled by a complex collaboration between chemokines and chemokine receptors.

Chemokines are often divided into two groups, they are either “homeostatic” or “inflammatory”. The homeostatic chemokines are constitutively produced in discrete microenvironments like the skin and the mucosa and maintain the physiological trafficking and positioning of cells throughout the immune system. In contrast the inflammatory chemokines are specialized in recruiting effector cells to inflamed tissues where they are produced by resident and infiltrating cells in response to inflammation (reviewed in [41, 42]). In summary, lymphocyte circulation can be divided into two parts: First, the continuous flow of lymphocytes between lymphoid organs and blood to maintain the balance of immune cells through out the organism, a phenomenon referred to as cell homeostasis. Second, the recirculation pattern that takes over after foreign antigens have been detected and a new series of complex changes attract antigen-specific B and T cells together to produce an adaptive immune response.

Homeostasis

The mechanisms that control the number of lymphocytes present in an organism during normal physiological conditions are poorly understood. The immune system continuously produces lymphocytes in excess but the total number of these cells in an individual is kept constant. Newly generated cells have to compete among themselves or with resident cells for survival. This competition can be defined as an interaction between two cell populations, in which the birth rates are reduced or the death rates are increased by the presence of the other population. Moreover the contest between these two cell populations may arise through different processes and the regulation of peripheral lymphocyte numbers might be controlled at several points, including rate of cell production, modulations in survival signals or peripheral cell division (reviewed in [43]). The result of this struggle is cell homeostasis, i.e. the internal equilibrium between cells in a cell population as well as the rates of cells in different cell populations of the immune system.

Resources in homeostatic control

Today evidence exists indicating that the role of different resources in lymphocyte competition is important. A resource may be defined as any factor that is advantageous for the cell survival or growth of a lymphocyte [44]. In a broad sense, any factor that is used by a cell and for that reason is unavailable for other cells, may be classified as a resource. The resources can be essential, complementary, substitutable, antagonistic or inhibitory [45]. Resources are abundant during for example the expansion phase of cells reconstituting a lymphopenic host but as the cell populations increase in size, competition for resources begin and cell growth reaches equilibrium. Therefore competition only occurs when resources are limited [46, 47] and the contest might contribute to the regulation of the size of the peripheral lymphocyte compartments [48]. In a host where the amounts of resources are in excess, a larger amount of newly generated T and B cells are able to survive.

Activated lymphocytes are major producers of their own resources and antigen stimulation increases resource availability by inducing lymphocyte activation and production of numerous cytokines. This means that lymphocytes can increase in number during the expansion phase of the immune response. After the elimination of the antigen the cytokine production decreases and at this reduced level of resources a minor number of lymphocytes can be maintained. Thus lymphocytes will die during the contraction phase of the immune response because essential resources are deficient (reviewed in [43, 49, 50]).

Apoptosis in homeostatic control

Programmed cell death (PCD) or apoptosis is an evolutionary conserved process that is important both during the development and protection of an organism since damaged cells are removed in a controlled way. Both the possible induction of inflammation and subsequent tissue destruction by necrotic cells are inhibited. In the immune system apoptosis regulates the repertoire diversity and maintains the size of the cellular compartments. Therefore apoptosis plays an essential role after antigen activation when the antigen has been successfully removed and the number of lymphocytes must be restored back to the steady state level. Thus homeostasis can only be maintained if there is a proper regulation of cell death. If the frequency of death is too low, cancer or autoimmunity can develop, while if the frequency of death is too high, the organism will suffer from leukopenia and immunodeficiency [51].

During apoptosis cells go through a series of biochemical processes and morphological modifications such as condensation of both the nucleus and the cytoplasm, which result in a size reduction of the cell. Furthermore, specific apoptosis markers are expressed on the cell surface and small apoptotic bodies develop (reviewed in [52]). This enables a controlled uptake of apoptotic cells by macrophages or other phagocytic cells (reviewed in [53]).

Two principal pathways of apoptosis induction are known; first the "extrinsic" pathway which is mediated by death receptors and secondly the "intrinsic" or Bcl-2 controlled pathway which is mediated via the mitochondria and controlled by the proteins in the Bcl-2 family (reviewed in [54]). The first pathway is activated when death receptors like tumor necrosis factor receptor 1 (TNF-R1) interacts with its soluble ligand TNF-α or when Fas (CD95/APO-1) binds its cell surface ligand FasL (CD95L) [55] (reviewed in [56]). The second pathway is activated by stimuli like environmental stress, DNA damage, reactive oxygen species, UV- and γ-irradiation, respiratory chain inhibitors, chemo-therapeutic drugs and withdrawal of growth factors [57-60] (reviewed in [61]). The activation leads via kinases and other proteins to cytochrome c release from the mitochondria and results in apoptosis induction. Both pathways induce apoptosis by activating different caspases which is a family of proteases that cleave their substrates and start up caspase cascades that potentiate the apoptotic response. Even though the death receptor pathway and the mitochondrial pathway often is presented as separate, several feed-back loops and crossover mechanisms exist between them (reviewed in [62]). Furthermore lymphocyte survival can be modified through the balance between different apoptotic and anti-apoptotic proteins in the cells.

The Bcl-2 family of proteins and their relatives are important modulators of apoptosis. The family contains both proteins that inhibit apoptosis (Bcl-2, Bcl-x_L, Mcl-1, A1) and proteins that promote apoptosis (Bax, Bak, Bad, Bid, Bik, Bcl-x_S). These different Bcl-2 proteins have varying distribution in the cytoplasm but after activation by an apoptosis signal they translocate to the mitochondria which is their proposed site of action (reviewed in [63, 64]). Finally one should mention that apoptosis can be regulated by the inhibitors of apoptosis (IAPs). These proteins have the capacity to regulate the activity of caspases (reviewed in [65]). All together, induction of apoptosis is a highly regulated process and both the function of the immune system and more specifically the regulation of cell homeostasis is dependent on the organism's capacity to control this process.

T lymphocyte homeostasis

The peripheral $CD4^+$ and $CD8^+$ T cell compartments are each divided into one naïve T cell compartment and one memory T cell compartment. These two compartments have their own autonomous homeostatic control that ensures the presence of T cells in the different subpopulations including naïve and memory $CD4^+$ and $CD8^+$ T cells. Furthermore $CD4^+$ and $CD8^+$ T cells can compensate for each other in the naïve compartments, thus an increase or decrease in either $CD4^+$ or $CD8^+$ T cells can be filled up by naïve T cells from the other phenotype even though they have distinct roles in the immune system (reviewed in [43, 66, 67]).

T cell homeostasis and the importance of MHC/TCR interactions

Today we know that naïve T lymphocytes are actively engaged in their own survival [49]. The recognition of major histocompatibility complex (MHC) proteins is required for the persistence of naïve T cells in the periphery. For the survival of $CD8^+$ T cells, continuous interactions between their TCRs and MHC class I molecules are required [68]. Furthermore the survival of $CD4^+$ T cells is dependent on the presence and recognition of MHC class II molecules [69-71]. However naïve T cells that have been exposed to antigens and differentiated into either $CD4^+$ or $CD8^+$ memory T cells do not need MHC class II or MHC class I molecules to survive [72, 73].

All together the size of the total T cell compartment is maintained at a remarkably constant level despite environmental antigenic stimulation and thymic output (reviewed in [43, 50, 66, 67]).

The T lymphocyte compartment

The production and migration of T cells from thymus out in the periphery modifies the survival terms for resident T cells. This modification is determined by the state of activation of the existing T cells. Naïve and tolerant T cells decayed while

memory cells persisted in constant numbers in the presence of thymic output. This phenomenon was due to a random substitution where resident T cells are competing with recent thymic emigrants for the same niches in the peripheral T cell pool [74]. This indicates that naïve T cells compete for their survival in an individual which already have a complete T cell repertoire. The size of the T cell compartment can also be affected by peripheral cell division. In a normal mouse the peripheral division is restricted but when peripheral T cells are transferred into a cell-deficient host the cells can expand considerably. It has been proposed that each transferred T cell can generate a progeny of about 10^{15} cells in a cell-deficient host [75]. The prevailing view about this expansion in cell-deficient hosts was that foreign antigens drive the expansion and that this expansion is intensified by increased availability of space plus contact with cytokines or infectious agents. However more recent studies have suggested that homeostatic proliferation can occur in the absence of cognate antigens and therefore the division might be a result of peripheral signals that promotes the recovery of the T cell compartment [76-82]. The proliferation of naïve T cells in lymphopenic hosts is often referred to as "homeostatic-driven proliferation (HDP) (reviewed in [67]).

Cytokines and T lymphocyte homeostasis

Cytokines are also implicated in the regulation of the T cell compartments affecting both cell survival and differentiation into effector cells. For example, injections of IL-7 into mice can increase the number of T cells in the periphery suggesting a role for this cytokine in the regulation of T cell survival [83]. Furthermore Lantz et al. have reported that memory and naïve $CD4^+$ T cells have in order to survive different requirements for the expression of the common γ-chain which mediates receptor signalling for IL-2, IL-4, IL-7, IL-9 and IL-15. The study showed that memory cells survived in the absence of the γ-chain while naïve cells died without it. These results suggest a role for cytokines in the survival of naïve $CD4^+$ T cells [84].

Costimulatory molecules might also play a role in T cell homeostasis. For example the CD152 (CTLA-4) molecule seems to be important since $CD152^{-/-}$ mice accumulate activated T cells in the peripheral lymphoid organs and die around four weeks of age due to massive tissue destruction [85, 86]. These results implicated that the homeostatic control fails from the beginning without CD152 but do not prove that CD152 plays a role in maintaining the steady state level in the T cell compartment. Furthermore it has been reported that in reconstituted irradiated mice, $CD28^{-/-}$ T cells are maintained less effective compared to wild type cells in the peripheral T cell compartment [87]. Finally Prlic et al. have reported that the CD28 molecule does not seem to be required for homeostatic expansion of T cells *in vivo* [88].

B lymphocyte homeostasis

Immature transitional B cells that have been generated in the bone marrow and successfully emigrated to the periphery compete for their survival against B cells that already exist in the host. The emigrant cells have a short life-span and only a few of them enters the long-lived mature peripheral B cell compartment. The production of B cells in the bone marrow is sufficient to populate the peripheral B cell compartments of two to three mice [89]. This suggests that the peripheral B cell numbers must be limited by mechanisms in the periphery and not by the rate of cells produced by the bone marrow. The half-life of mature peripheral B cells is still a matter of debate however *in vivo* labelling with BrdU determined the life time to be between several weeks to months [90]. Furthermore in the absence of B cell export from the bone marrow the life time was determined to be around 4,5 months [91]. Finally, similarly to the T cell compartments, the size of the peripheral naïve and memory B cell compartments are independently regulated [89].

B lymphocyte homeostasis and the importance of BCR interactions

The maintenance of naïve peripheral B cells appears to involve interactions between the B cell receptor (BCR) and unidentified ligand(s) that provide the B cells with survival signals [92]. After the generation of B cells in the bone marrow, naïve B cell survival is associated with peripheral selection of B cells that express particular V_H-gene families [93, 94]. Thus the recognition interactions related to B cell survival might be exclusively V_H-gene mediated and without the requirement for the full antigen binding site. The survival of memory B cells suggest that antigen is required at an early state of cell activation and during selection of B cells in the germinal centers but recent data suggest that once the cells have acquired a memory phenotype they do no longer require recognition of immunizing antigen to survive [95].

Cytokines in B lymphocyte homeostasis

Recently the cytokine BAFF (B-cell activating factor) which is produced by myeloid cells was identified as an enhancer of B cell survival. The lack of BAFF reduces the peripheral B cell compartment and BAFF-deficient mice show depletion of peripheral B cells and negligible IgM responses [96]. Furthermore BAFF expands the number of B cells *in vivo* by increasing their survival, suggesting an important role for BAFF in regulating B cell homeostasis (reviewed in [97]). In addition, the cytokine IL-6 might influence the survival of plasma cells since plasmacytomas and multiple myelomas require IL-6 to survive [98]. The role of costimulatory molecules in B survival is not well investigated but Yin et al. showed that the expression of the CD40 ligand on T cells might play a role in the maintenance of B cell homeostasis [99].

Summary of lymphocyte homeostasis

All together the size of T and B lymphocyte populations is independently regulated in adult mice. The number of mature B cells is approximately the same in normal

mice and in mice that lack T cells [100]. Mice that lack B cells contain similar numbers of T cells to that of normal mice [101]. Moreover B and T cells occupy different regions in the secondary lymphoid organs. Thus B and T cells belong to different guilds and need largely different resources to survive. However some resources like hormones and mitogens are functionally pleiotropic and changes in the access of these common substances will modify overall cell numbers. In summary, the survival of lymphocytes relies on cell/ligand interactions, the availability of resources and the nature and number of competing rivals.

Leukocyte homeostasis

As already mentioned above lymphocyte numbers are kept at a fairly constant level in the periphery and the life-span of other leukocytes together with their out put rate is an important part of the homeostatic control. The mechanisms that control homeostatic regulation of other leukocyte populations have also been examined. Below I will briefly summarize what is known about their life-span.

The monocytes represent five to ten percent of the leukocytes in the peripheral blood of humans, mice and rats. The cells migrate from the bone marrow into the blood where they circulate for about a day and then migrate to different organs to become organ specific macrophages such as Kuppfer's cells in the liver or alveolar macrophages in the lung or ordinary tissue macrophages in the connective tissues. Even though resident macrophages can be quit long lived (years), monocytes are produced in excess and most of them die after induction of apoptosis. Thus the compartment of macrophages in an organism during homeostasis is controlled by continued output of monocytes from the bone marrow together with a controlled cell turnover orchestrated by apoptosis (reviewed in [102, 103]).

Neutrophils have the shortest life-span among leukocytes since they die by spontaneous apoptosis one to two days after migration from the bone marrow to the blood. The large turnover of neutrophils is compensated by a continuous output of

cells from the bone marrow and the life-span of a neutrophil can be modulated by various molecules [104].

Dendritic cells (DCs) are a heterogeneous group of antigen presenting cells (APCs) distributed through out various tissues in the body [105]. It has been suggested that DCs do not divide in the periphery and therefore the cell compartment must be continuously replenished by the bone marrow [106]. DCs in the lymph nodes are currently divided into at least four different subsets based on their origin, surface molecule expression and function. Further, the life-span of these subpopulations varies. The DCs that migrate from the bone marrow to the lymph node have a turnover time of about ten days while the DCs that are resident in the skin and migrate from the skin into a lymph node are more long lived [107]. All together the output and turn over rates of all leukocyte populations are highly regulated while the life-span among different leukocytes varies.

Lymphocyte activation

Various stimuli can activate T and B lymphocytes and the induced molecular mechanisms ending in cell differentiation involve a large number of molecules. In this chapter I will shortly describe the main signalling pathways for T and B cells. Two schematic pictures of the described pathways are shown in Figure 1 (T cell activation) and Figure 2 (B cell activation).

Activation of T lymphocytes

For the activation of naïve T cells two signals are needed. Firstly, a signal delivered via the TCRs after ligation with antigen-bound MHC molecules on an APC and secondly a signal delivered via costimulatory molecules. The $CD4^+$ T cells recognize antigens on MHC class II molecules and $CD8^+$ T cells recognize antigens on MHC class I molecules. The most important costimulatory molecule for the activation of T cells is CD28 which binds to the B7 molecules on the APCs (reviewed in [108]). However, T cells also express other molecules which are referred to as costimulatory. These molecules often have adhesion properties to ensure the contact between APCs and T cells and thereby a successful delivery of the second signal. The activation step involves the formation of a supramolecular activation cluster (SMAC) on the T cells [109, 110] where costimulatory molecules and the TCR are located in the center of the SMAC surrounded by a ring of adhesion molecules. This formation ensures a proper localization of both TCR and costimulatory molecules during early activation and helps the cell to reorganize the actin cytoskeleton [111-113]. Furthermore specialized microdomains in the cell membrane called lipid rafts mediate the signalling by the receptor and are said to both recruit and exclude proteins in this process (reviewed in [114-116]). After the triggering, intracellular biochemical events involving a large amount of molecules lead to the activation of the nuclear factor of activated T cells (NF-AT) which translocate to the nucleus and plays an important role for the transcription of the IL-

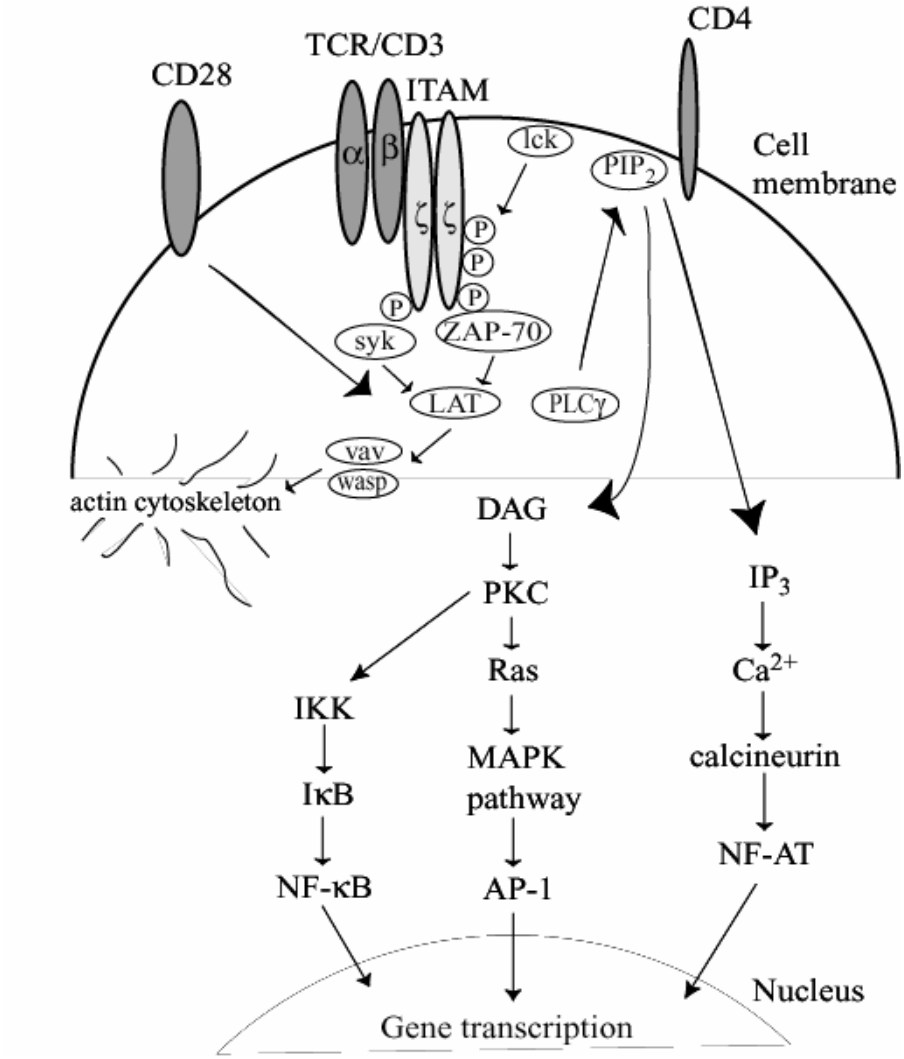

Figure 1. A schematic description over some of the biological events that occurs during T cell activation in lymphoid tissue.

2 gene [117]. Furthermore the transcription factor complex AP-1 becomes activated through the so called mitogen-activated protein kinase (MAPK) pathway which is

among the most common signalling pathways in eukaryotic cell regulation (reviewed in [118]). In addition the transcription factor nuclear factor κB (NF-κB), which will be discussed in a later section of this thesis, becomes activated. Together NF-AT, AP-1 and NF-κB activate transcription of several important genes encoding both cytokines and cell surface molecules. Following the activation signal, the T cells enter the cell cycle and starts to produce cytokines, especially IL-2 which is important in both the continued activation of T cells by autocrine loops and for stimulating cell proliferation (reviewed in [119]). The T cells differentiate and start to express new gene patterns that are important for their effector function and at this stage they may be referred to as immature effector cells. The $CD4^+$ helper T cells (Th) continue to differentiate into either Th1 or Th2 cells which are distinguished by their cytokine profile and their function. Th1 cells produce interferon-γ (IFN-γ) and IL-2 and are important for the clearance of intracellular pathogens such as bacteria, yeasts, viruses and parasites. The Th1 response is often associated with cell mediated immunity and includes increased microbicidal activity of macrophages, activation of NK cells and CTLs that produce IFN-γ and perforin. In contrast, Th2 cells produce IL-4, IL-5 and IL-13 and are important for the clearance of helminths and other extracellular parasites. Furthermore the Th2 response mediates humoral immunity and activates B cells to produce antibodies. In addition mast cells and eosinophils are activated. The factors that control whether Th cells proliferate into either Th1 or Th2 cells are not fully understood. However, factors like antigen dose, antigen form, TCR affinity and costimulatory interactions might influence the decision. Cytokines are also important factors for driving Th cells into either Th1 or Th2 cells and IL-12 is dominant for inducing Th1 cell responses while IL-4 directs towards Th2 responses (reviewed in [120, 121]).

Naive $CD8^+$ T cells differentiate into cytotoxic T cells (CTL) after activation and start to secrete soluble lytic proteins like perforin and granzymes towards infected cells that express antigen on their MHC class I molecules. This response leads to lysis and nuclear degradation of the infected cells. Furthermore CTLs express Fas

ligand molecules which can induce apoptosis in target cells that bears the Fas molecule on the cell surface. Finally, as mention above CTLs produce cytokines like for example IFN-γ (reviewed in [122]).

Activation of B lymphocytes

Two different mechanisms can initiate B cell activation, either activation via Thymus-dependent (TD) or activation via Thymus-independent (TI) antigens. The difference between TD and TI antigens is that the former need help from T cells to activate B cells while the latter can activate B cells independently of T cells by cross-linking their BCRs. Furthermore TI antigens are subdivided into TI-1 and TI-2 antigens. TI-1 antigens are non-protein bacterial molecules such as bacterial lipopolisaccharide (LPS) which at high concentrations is a polyclonal stimulator of B cell activation *in vitro*. LPS binds to the Toll Like Receptor 4 (TLR-4) which is part of a pathogenic protection system and currently ten different TLR's, that all recognize various microbial products, are known in humans (reviewed in [123, 124]).

TI-2 antigens are highly repetitive molecules like for example bacterial polysaccharides that can crosslink BCR molecules. This group of antigens only stimulate mature B cells and are unable to initiate a response in neonatal mice. In contrast, TI-1 antigens stimulate responses in both mature and neonatal B cells (reviewed in [123, 125]). Even though this general division indicates that TI antigens activate B cells in the absence of T cell help and neither high-affinity antibodies nor memory B cells are generated during these responses exceptions exists and studies demonstrating both memory B cell and germinal center formation induced by TI antigens have been presented [126-129].

The TD antigens are internalized after ligation to BCRs and the antigen-BCR complex is transported to MHC class II-enriched compartments (MIIC) and processed into peptide fragments (reviewed in [130]). These processed peptides are

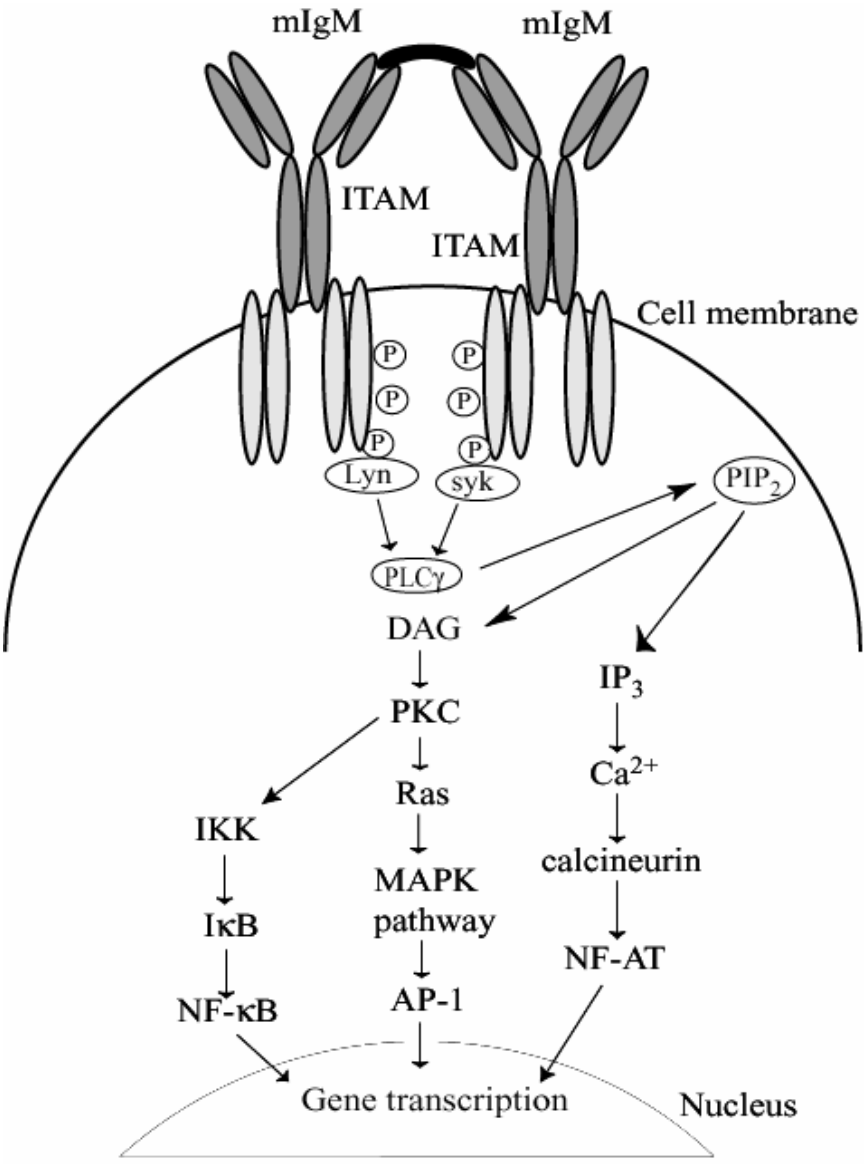

Figure 2. A schematic description over some of the biological events that occurs during B cell activation in lymphoid tissue.

associated with the MHC class II molecules and shuttled to the cell surface. At the same time an increased expression of cell surface molecules, important for interaction with T cells, are expressed on the B cell surface. Finally, the T-B cell interaction activates signalling pathways in the B cell that initiate the formation of germinal centers, clonal expansion, somatic mutation, immunoglobulin class switching and plasma cell differentiation.

All together BCR ligation activates a cascade of down stream signalling pathways that leads to recruitment of proteins, activation of GTP-binding molecular switches, phosphorylation and gene transcription. Important transcription factors involved in the regulation of the gene transcription are among others NF-AT and NF-κB (reviewed in [131]).

The transcription factor NF-κB

Nuclear factor κB (NF-κB) belongs to a family of transcription factors that have central roles in both innate and adaptive immunity. It was first thought that NF-κB only existed in B cells when it was described for the first time by Sen and Baltimore in 1986 [132]. However, more recent studies have shown that the NF-κB family is present in almost every cell throughout the body. In lymphocytes, NF-κB regulates cell survival, proliferation, activation and production of various cytokines through gene regulation (reviewed in [133]). Several members of the family have been described including, RelA (p65), NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), c-Rel and RelB. These proteins are structurally conserved containing a Rel homology domain (RHD) responsible for DNA binding, dimerization and interaction with inhibitory proteins. All family members can form homodimers and heterodimers with each other, except RelB, enabling them to bind to DNA. The general transactivating NF-κB form in many cell types is a heterodimer of Rel A (p65) and p50 subunits. Furthermore, some of the dimers that appear i.e. the p50/p50 combination can suppress transcription even though binding of NF-κB proteins usually promotes gene transcription. This is probably because the p50/p50 homodimer lacks a transcriptional activation domain (Reviewed in [134, 135]).

Activation of NF-κB

The transcription factor NF-κB can be activated by many different stimuli including ligation to the TCR or BCR, pro-inflammatory cytokines i.e. TNF-α and IL-1, phorbol esters, LPS, double stranded RNA, the TAX protein of HTLV-1, cell stress and viruses (reviewed in [135, 136]). These different stimuli use various intracellular signalling pathways for activating NF- κB even though most pathways seems to converge at the point where the inhibitory IκB proteins are phosphorylated

by the IKK complex. The phosphorylation of inhibitory proteins results in release of NF-κB proteins in the cytoplasm and makes translocation into the nucleus possible. These events are schematically described in Figure 3.

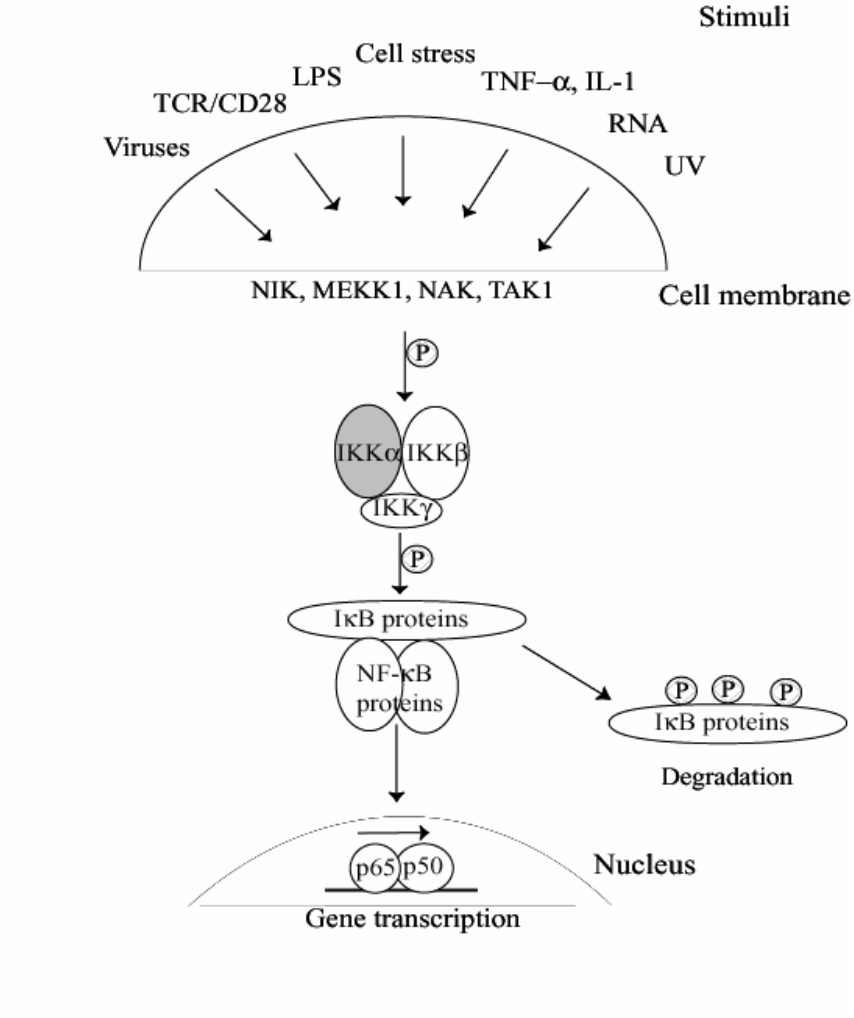

Figure 3. A schematic description of stimuli, kinases and inhibitory proteins involved in activation of NF-κB.

The IKKs and IκB proteins

In normal cells, activation of NF-κB is mainly controlled by various IκB inhibitory proteins. The IκB proteins are phosphorylated by the multiprotein IκB kinase (IKK) complex that contains two homologous catalytic subunits the IKKα and IKKβ. Furthermore the regulatory subunit IKKγ, also called NEMO, and the recently defined IKKε protein are members of this complex (reviewed in [137, 138]). The IKKα and IKKβ proteins were the first IκB kinases to be defined [139-142] and the regulatory IKKγ subunit binds to these [143-145], forming a high molecular weight complex (review in [146]). The IKK complex is needed for IκB phosphorylation and this is a converging point in NF-κB activation. However, the molecular mechanism(s) that transmits signalling from the cell surface and initiates IKK activity is not well understood. Today several upstream kinases that activate IKK's have been identified including MEKK1, MEKK2, MEKK3, NIK, TBK1, TPL2 and TAK1. Furthermore it is clear that various receptors, cell types and stimuli have the ability to develop specific pathways that mediates IKK activation (reviewed in [133, 137, 147]). Recently another member of the IKK proteins was discovered, the IKKε which seems to possess the qualities of an IκB kinase even though the activator still is unknown [148, 149].

Today several prototypic isoforms of IκB proteins are known, including IκBα, IκBβ, IκBγ IκBε (two transcripts) and Bcl-3. Furthermore the precursor proteins p100 and p105 belong to this family. These inhibitory proteins carry multiple ankyrin repeats that mediate protein interactions and retain the NF-κB/Rel dimers inactive in the cytoplasm. After activation, IκB proteins are degraded and free NF-κB proteins can translocate into the nucleus to promote transcription. This is a temporary state since the inhibitory protein gene IκBα itself is a target for NF-κB induced transcription and its re-synthesis acts as a regulatory feed-back loop that inhibits further NF-κB activity [150, 151]. The IκBα protein has a nuclear import sequence that makes it possible for the newly synthesised IκBα to enter the nucleus

and transport NF-κB out to the cytoplasm, where it is inactivated [152, 153]. Thus IκBα is thought to be important for transient NF-κB responses while IκBβ is thought to be important for maintaining NF-κB activation since IκBβ bindes to the same NF-κB proteins as IκBα but is slowly degraded compared to the IκBα protein [154]. In addition the IκBβ gene is not under the control of the NF-κB transcription factor and production of IκBβ protein is therefore not as fast as production of IκBα protein after NF-κB activation [155] (reviewed in [133, 134, 147, 156]).

NF-κB and cell cycle regulation

NF-κB has a central role in the regulation of cell cycle progression and controls several steps in the cycle (reviewed in [157-159]). In 1991 Baldwin et al. showed that NF-κB was induced in mouse fibroblasts during the G_0 phase to the G_1 phase transition [160]. Recently it was reported that inhibition of NF-κB activity impaired cell cycle progression in glioma cells and retarded the transition between the G_1 phase and the S phase in HeLa cells [161, 162]. Furthermore there was a cell cycle block induced between the G_1 and S phase in HeLa cells when the NF-κB protein c-Rel was overexpressed [163]. In accordance with this result overexpression of the p65 protein also caused a G_1 block and apoptosis in pro-B cells [164]. Furthermore one report indicating the need for NF-κB activity between the G_2- to M-phase transitions in an epithelial cell line was recently published [165].

NF-κB and apoptosis

Apoptosis can also be regulated by the activity of NF-κB and there seems to be a clear connection between these two events in the cell since activation of apoptosis almost always induces NF-κB activity. Thus, some of the NF-κB and IKK knock out mice die early because of massive liver apoptosis and ligation to TNF receptors either activates inflammation or induces apoptosis via the extrinsic pathway [166-171]. In addition activation of NF-κB leads to transcription of both inflammatory

and anti-apoptotic genes and lack of NF-κB activity often results in apoptosis. However, evidence exists suggesting that NF-κB activation can regulate apoptosis both positively and negatively. The decision whether NF-κB activity will result in apoptosis induction or prevention depends among other things on the cell type and the activation stimuli (reviewed in [158, 172, 173]).

NF-κB and inflammation

NF-κB plays an important role in inflammation where its activation under inflammatory conditions works as an autoregulatory feedback loop since pro-inflammatory cytokines activates NF-κB resulting in transcription of TNF-α and IL-1 cytokine genes. This mechanism ensures that the inflammatory signal continues and that inflammatory responses develop. In addition, NF-κB influences the expression of adhesion molecules such as E-selectin, the vascular cell adhesion molecule (VCAM) and the intracellular adhesion molecule (ICAM) [174] which are important for the regulation of leukocyte migration into inflamed areas. Furthermore NF-κB has been implicated in different inflammatory diseases like rheumatoid arthritis, multiple sclerosis, asthma, inflammatory bowel disease and atherosclerosis (reviewed in [175, 176]). Hence, several drugs that are currently used to treat inflammatory conditions have effects on NF-κB activity (reviewed in [177]).

NF-κB and drugs

The central role of NF-κB in cell regulation and inflammatory processes makes it into one of the most attractive targets for developing new immunosuppressive and anti-inflammatory therapies. Today several drugs that inhibit NF-κB activity in some way are used to treat various inflammatory diseases (reviewed in [178]). These are for example non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin. The active compound in aspirin is sodium salicylate originating from

salicylic acid, which is present in many plants and inhibits NF-κB activity [179]. This finding functionally linked the inhibitory effects of salicylic acid on prostaglandin production (reviewed in [180]) with an important transcription factor that is involved in inflammation and suggested a mechanism for the modulation of the inflammatory response. Furthermore the aspirin induced inhibition of NF-κB had a protective function in neurons [181]. The observed inhibition of NF-κB by aspirin and sodium salicylate was later ascribed to inhibition of IKKβ kinase phosphorylation thus, no IκB degradation occurred and the NF-κB proteins could not enter the nucleus [182]. Other NSAIDs that inhibit NF-κB activity by affecting IKKβ are Sulindac [183] and cyclopentone prostaglandins such as PGA1 and 15d-PGJ2 [184, 185] . In addition, Thalidomide an anti-nausea drug also has been reported to inhibit NF-κB by suppressing IKK activity [186].

Other substances not yet available as therapeutic products but still known to effect NF-κB activity are different plant derived compounds like for example the EGb 761 extract derived from *Ginko biloba*. In a recent report it was shown that this extract diminished LPS induced NF-κB activity and inhibited the production of the pro-inflammatory cytokine TNF-α both *in vitro* and *in vivo* [187]. The substance curcumin derived from the plant *Curcuma longa* inhibit NF-κB activity [188-190] and it has shown beneficial effects in a mouse model against induction of inflammatory bowel disease [191]. In addition curcumin has shown potential against urinary bladder cancer, uterine cervical intraepithelial neoplasm and intestinal metaplasia of the stomach in humans [192]. Thus, curcumin might become useful for treating various human disorders in the future. Finally, the caffeic acid phenethyl ester (CAPE) present in *Apis mellifera* and avicins a family of triterpenoid saponins isolated from *Acacia victoria* are other compounds reported to inhibit NF-κB activity [193-195] and (reviewed in [196]).

In a recent study, Sandoval-Chacon et al. observed that an extract from the vine *Uncaria tomentosa* inhibited NF-κB activity [197]. Furthermore it was reported that

the production of the pro-inflammatory cytokine TNF-α was inhibited by *Uncaria tomentosa* extracts [198]. In paper II of this thesis we showed that the water derived *Uncaria tomentosa* extract C-Med 100® inhibited the activity of NF-κB in a reporter gene assay and cell proliferation in mitogen stimulated splenocytes [199]. Our results were in accordance with the previous study made by Sandoval-Chacon et al. [197]. In addition, paper III of this thesis reported that the isolated biologically active component from C-Med 100®, quinic acid also inhibited NF-κB activity. We showed that at least one mode of action for quinic acid was to inhibit NF-κB activity by retarding the phosphorylation of the IκBα protein a mechanism that was not observed when the crude C-Med 100® extract was investigated.

Taken together, there are a number of plant products inhibiting NF-κB activity that might become useful in the development of new therapeutic agents for treating various inflammatory disorders.

Finally, as mentioned above NF-κB is expressed in a wide variety of cells and different tissues controlling various processes, thus an overall inhibition of its activity would probably cause major harmful side-effects for an organism. Therefore it is important to elucidate the mode of action of new interesting compounds that inhibit NF-κB activity. Pharmaceutical companies are currently developing compounds that selectively inhibit NF-κB by for example blocking the DNA-binding activity of individual NF-κB proteins. This strategy is used to avoid side-effects that compromise normal host functions during treatment (reviewed in [133]).

The inflammatory response

Invasion by foreign material like bacteria, parasites and viruses induces inflammation. Thus the inflammatory response is an important reaction that protects us and it is characterized by redness, heat, swelling, pain and loss of function. Furthermore it can be triggered by irritation and injury. The inflammatory response leads to successful clearance of infection and healing of the injured tissue. However, in some cases the infection is not cleared and a chronic inflammation develops which causes severe tissue damage and may lead to the development of granulomas (reviewed in [200]).

Tissue injury often causes an inflammatory response. It is important for the organism to detect if there are accompanying microorganisms in a wound and to prevent the spreading of these, even if the consequence is further tissue damage. Therefore tissue destruction by itself initiates two types of activation signals in the immune system; (i) response to pain by neurons that triggers release of bioactive peptides [201] and (ii) substances such as the transcription factor HMGB1 (high mobility group 1) and mitochondrial peptides are released from the broken cells [202, 203] (reviewed in [204]).

Mast cells and macrophages located in the tissue respond to the first signals and start to produce histamine, cytokines, proteases and chemokines. Among other compounds histamine causes vasodilatation and extravasation of fluid which makes it possible for proteins and cells to enter the tissue. Furthermore chemokines recruit neutrophils and monocytes to the inflammatory site. The neutrophiles migrate from the blood into the inflamed tissue where they either engulf microorganisms by phagocytosis or release toxic mediators and cytokines. The pro-inflammatory cytokines IL-1, IL-6, TNF-α and INF-γ are important in the inflammatory response and withdrawal of these cytokines and production of anti-inflammatory cytokines like IL-4, IL-10, IL-13, IFN-α and TGF-β resolves the inflammatory response. The accumulation of cells and fluid in the tissue creates the characteristic markers of

inflammation; swelling, heat, redness and pain. The macrophages and neutrophils are the principal inflammatory cells and lymphocytes participate only later in the inflammatory response (reviewed in [205-209]).

Inflammation and drugs

Current therapeutic approaches for treating rheumatic diseases focus on preventing the pro-inflammatory cytokine TNF-α by administration of neutralizing antibodies or antagonists [210] (reviewed in [211]). However, it is interesting to speculate that cytokines involved in the resolution of inflammation might be suitable targets for treating chronic inflammation in the future. Long-term inflammatory responses which often are associated with allergies, autoimmune diseases, some microbial infections, transplants and burns are complicated to cure and various approaches have been investigated. Examples of therapeutic agents used in different inflammatory models are; (i) agents that reduce leukocyte extravasation such as neutralizing antibodies to the leukocyte functional antigen (LFA)-1 or the intercellular adhesion molecule (ICAM)-1 [212-215], (ii) corticosteroids and (iii) non-steroidal anti-inflammatory drugs (NSAIDs) already mentioned in the NF-κB and drugs section.

Among these, corticosteroids are potent anti-inflammatory agents with various effects including inhibition of chemotaxis, phagocytosis, cytotoxicity and down regulation of cytokine gene expression (reviewed in [216]). Furthermore studies have shown that corticosteroids can inhibit IL-2 gene transcription by interfering with transcription factors like NF-AT and AP-1 [217]. In addition, these steroid hormones induced the transcription of the NF-κB inhibitor IκBα protein thereby inhibiting NF-κB activity [218, 219]. Finally corticosteroids inhibit adhesion and migration of inflammatory cells by down regulation of adhesion molecules such as ICAM-1, LFA-1 and E-selectin or by interfering with migration itself [220-222].

All together corticosteroids have a major role in therapeutic treatments of inflammatory conditions today. However, undesirable side-effects such as

weakened bones and abnormal fat accumulation have been ascribed to these compounds especially after long term treatment, which is required for many of the conditions that they are prescribed against. Thus, new anti-inflammatory agents with less harmful side-effects are still needed for treating inflammatory disorders.

Medical plants and phytopharmaceuticals

Herbal remedies since ancient times

Mankind has always searched for methods that treats disorders such as pain, inflammation and other diseases and for this purpose medical plants and herbal preparations have been used since ancient times. One evidence was found in a grave of a Neanderthal man buried 60 000 years ago where pollen analysis indicated that the plants buried with him were all of medical value. In the earliest known medical document, a 40 000-year old Sumerian writing, plant remedies were described for various illnesses. Medical plants in ancient China have also been recorded and old documents are a source of information about plants in early medicine. Thus, in a pharmacopoeia published around 1600 B.C. thousands of herbal cures were mentioned. Herbal medicine in Ancient Egypt and India goes back thousands of years describing medical plants, especially for example in the collection of Hindu sacred verses (Reviewed in [223]).

The foundation of Western-type medicine is often attributed to ancient Greece and the physician Hippocrates, who used various herbal remedies for treating diseases. The Greek Dioscorides described over 600 plants having medical value and included both illustrations of the plants together with directions of preparations, uses and side effects. Plants from the New World became available for Europeans after Columbus journey and for centuries, medicines in Europe were based on herbs. However, during the eighteenth century herbalism was diminished due to the great interest and progress of scientific knowledge.

The nineteenth century scientists began to purify extracts from medical plants and for example morphine was isolated from the opium poppy and quinine from *Cinchona* bark. During this century the first purely synthetic drugs based on natural products were produced and one of them was salicylic acid, an active ingredient in a number of plants with pain-relieving qualities (Reviewed in [223]).

Today populations in developing countries continue to use traditional medicine as treatments for primary medical problems. Even in the Western world there is a renewed interest for medicinal plants and the use of herbal products has grown dramatically. Recent estimates among people with cancer indicated a general use of medical remedies among 13-60 percent of the patients [224].

Most medical plants come from the tropical areas of Asia, Africa, South America and Caribbean, where they often are used both as food and medicine. The material is supplied through collection both from wild populations and from cultivations (Reviewed in [223, 225]). Thus the tropical rainforests that covers 12 percent of the earth are a vital source of plants that can be used for medical preparations. However, only one percent of the plants have been tested for pharmaceutical properties although the fact is that at least 25 percent of the modern medicines descend from plants. Furthermore about 35 000 identified plants out of several hundred thousands are used worldwide for medical purposes and in the future the role of plants in medicine might be of increased importance for health care systems (Reviewed in [223, 226]).

Important medicines originally isolated from plants

Some of the most used medical substances today such as atropine, morphine, digitoxin/digoxin, quinine, taxol, vincristine and vinblastine were originally derived from plants. Here a synopsis of these medically active ingredients is presented in order to show the diversity of plants as sources for pharmaceutical development.

(i) Atropine is extracted and isolated from the plant, deadly nightshade or *Atropa Belladonna*, which has been cultivated on almost all continents. The plant has been used alone or in combination with other atropine-containing herbals for millennia as an anodyne, soporific and hallucinogenic agent. Today it is still important because of the content of the medical substances atropine and scopolamine which are derived from the roots or the leaves [227]. Thus atropine is an anticholinergic drug

and has been routinely used of physicians to induce pupil dilatation during eye examinations but also for treating eye inflammations (Reviewed in [228]).

(ii) The poppy, *Papaver somniferum,* and its product opium has been known for many centuries. The compound opium prepared from the latex of the poppy is believed to have been used since prehistoric times both as a pain-relieving and as a soporific drug. Before the nineteenth century the active compound(s) were not known and it was almost impossible to apply the substance in exact doses. However, the developments in chemistry and medicine during the nineteenth century led to the discovery of the active ingredients of poppy and shortly after synthetic substitutes were provided [229]. Morphine was isolated from opium in 1805 and since then, it has become the most widely used analgesics for severe pain due to its effects on the central nervous system, where it binds to the endorphin receptors [230]. Heroine is the hydrochloride of diacetylmorphine and was discovered by acetylation of morphine. Heroine was shown to be more effective than morphine in pharmacological studies and the earliest results was so promising that it was considered a wonder drug. However, repeated treatment with heroin resulted in a development of tolerance and the patients became addicted to heroin [231].

(iii) The cardiac glycosides, digitoxin and digoxin, are extracts from the leaves of the common foxglove plant, *Digitalis purpurea*. They have been used to treat patients with congestive heart failure for more than 200 years but the safety and efficiency of these drugs has always been debated because of their toxicity. Today digoxin is the most commonly prescribed of the two glycosides and the use of the drug in treatment of heart failure is now indicated almost only for the control of a fast heart rate in patients with atrial fibrillation (reviewed in [232]).

(iv) Quinine is an alkaloid extracted from the bark of the *Cinchona* tree which was brought to Europe from Peru. During the nineteenth century isolation of quinine and other cinchona alkaloids was achieved and since then quinine has been the source of aminoquinoline drugs such as chloroquine. Today chloroquine is the first

drug of choice for treatment of malaria caused by the Plasmodium vivax and it continues to be the most widely used antimalarial drug in the world due to its low cost and good tolerability [233] (reviewed in [234]).

(v) Drugs that have been derived from plants are also used in cancer therapy and today seven anticancer drugs approved for commercial production exist. Three of these, *paclitaxel* (Taxol), *vinblastine* (Velbe) and *vincristine* (Oncovin) are antimitotic and influence the cell cycle in both the G_1- and the S-phase rendering cell division impossible.

Paclitaxel (Taxol) was derived from the Pacific Yew tree, *Taxus brevifolia* and today it is the first drug of choice for treatment of several tumourous cancers including breast cancer and ovarial cancer (reviewed in [223]).

The alkaloid *vinblastine* and the related alkaloid *vincristine* were both derived from the *Catharanthus roseus*, which were found in the Madagascar periwinkle in the 1950s. These alkaloids have been of great pharmaceutical importance for treatment of many forms of cancers such as malignant melanoma, malignant lymphoma and leukaemia for more than 40 years. Thus, since the discovery of *vinblastine* the survival rate of childhood leukemias has increased by 80 percent. However, the plant contains a low amount of alkaloids and therefore it has been an important pharmaceutical development to establish alternative production of these compounds, for example by *in vitro* cultures of *Catharanthus roseus* cells (reviewed in [223, 235]).

Herbal preparations with immunomodulatory effects

Commonly used herbs like, *Echinacea purpurea*, *Panax ginseng*, *Astragalus membranaceus, Hypericum perforatum* and *Ginko biloba* have been shown to possess properties that modulate the immune system in various ways [187, 236] (reviewed in [237]). Some of these effects are for example, increased phagocytic activities and/or effects on the cell-mediated and humoral immunity but also selective stimulation of cytokine production [238] (reviewed in [239]).

(i) *Echinacea* is an herb used by the Plains Indians (US) for a variety of disorders and recently it has been shown to influence immunological functions both *in vivo* and *in vitro* [240-242]. Gan et al. studied the effects of soluble extracts from *Echinacea purpurea* on natural killer (NK) cells *in vitro*. Short time exposure to low concentrations of *Echinacea* extract potentially activated NK cells. Thus the frequency of CD69 expressing cells increased, the mean fluorescence intensity of $CD16^+$ and $CD16^+CD56^+$ cells was augmented and the cytotoxicity was enhanced one hundred percent [243]. Furthermore Cundell et al. performed a double-blind placebo controlled study to investigate if *Echinacea* could affect white blood cell counts, phagocytic activity and IL-2 concentrations *in vivo*. In animals supplemented with *Echinacea,* the number of circulating white blood cells increased significantly during the first two weeks of treatment. There was an immediate significant increase in the percentages of lymphocytes and monocytes while there was a significant decrease in the percentage of circulating neutrophils. Furthermore oral ingestion of *Echinacea* resulted in a significant increase in systemic blood IL-2 levels during the last five weeks of treatment. However there was no difference in the phagocytic function and no change in the basophil numbers [244].

(ii) *Panax ginseng* is one of the most popular herbs in the world and it has been used in traditional medicine in China for thousands of years. The active compounds in the plant are ginsenoides of which there are more than two dozen with low toxicity. In laboratory and clinical analyses the most notable characteristics of *Ginseng* are immune system modulations, antistress activities and antihyperglycemic activities (Reviewed in [245]). In a study See et al. treated peripheral blood mononuclear cells (PBMC) from normal individuals and from patients with either acquired immunodeficiency syndrome (AIDS) or chronic fatigue syndrome (CFS) with extracts from either *Echinacea purpurea* or *Panax ginseng*. The results indicated that both extracts enhanced the cellular immune function of PBMC in normal subjects as well as in patients with AIDS or CFS. The

antibody-dependent cell-mediated cytotoxicity (ADCC) against human herpes virus 6 infected H9 cells was also investigated. The extract *Echinacea* significantly increased ADCC in all three groups and *Ginseng* significantly increased ADCC in cells from normal individuals and at a 10-fold higher concentration in cells from AIDS or CFS patients [246].

(iii) *Astragagalus membranaceus* is an important herb in Chinese medicine and it has usually been applied to treat fatigue, loss of appetite and diarrhoea. Recent studies have shown that water-soluble extracts of *Astragagalus* were able to stimulate the proliferation of splenic lymphocytes and increase the mRNA expression of the IL-1α and IL-12p40 cytokines [247]. Furthermore Song et al. showed an increase in activated B cells after *in vivo* treatment with *Astragalus* together with ovalbumin (OVA) immunization [248] (reviewed in [239]).

(iv) St John´s wort, *Hypericum perforatum*, has mostly been studied for its effects on depression and not for immunostimulating properties (reviewed in [249]). In a study of Wilasrusmee et al. this herb among others was tested *in vitro* in a mitogen stimulated lymphocyte proliferation test and in mixed lymphocyte cultures (MLC). The results showed a significant increase in the [^{3}H] thymidine incorporation in both assays [238].

(v) The *Ginko biloba* extract EGb 761 is the most commonly prescribed herbal preparation in France and Germany and it is used to treat cardiovascular, peripheral vascular and cerebrovascular diseases. EGb 761 is composed by terpenoids and flavonoid glycosides such as quercetin (reviewed in [250]). In the mouse, Wadsworth et al. showed that pre-treatment with the EGb 761 extract inhibited LPS-induced increase in TNF-α in serum. Furthermore the EGb 761 extract diminished NF-κB activity and inhibited activities in the MAPK pathway *in vitro* [187].

Species from the Rubiaceae family

Uncaria tomentosa or Cat´s claw belongs to the family *Rubiaceae*, subfamily *Cinchonoideae*, and genus *Uncaria*. These species grow in tropical regions and consist of climbing woody vines with hook-like thorns. Currently at least 35 species of *Uncaria* are known, even though only a few of them are of interest from a pharmaceutical perspective. Phillipson et al. presented in 1978 [251] (reviewed in [252]) data about alkaloids extracted from approximately 400 small samples of *Uncaria* collected from a wide geographic range representing all the known species. About 40 different alkaloids were identified and they were mainly of the heteroyohimbine- and corresponding oxindole-types. Following the work of Phillipson et al. in 1978, terpenoids based on urosolic, oleanolic or quinovic acid structures, flavanoids based on oxygen containing phenolic acids and quinic acid also have been identified as active ingredients of various extracts of the *Uncaria* species [253, 254] (reviewed in [252]). Many novel bioactive compounds have been isolated, that vary greatly in their solubility in polar solvents such as water. Although biological activities of purified components could be assigned to individual chemical structures, their overall contribution to *Uncaria* crude extracts that have been marketed for healthcare efficiency has not to our knowledge been determined in any case except for C-Med 100® [253]. On the other hand, extracts from *Uncaria* have been isolated from various parts of the plant like the stem, bark, root, leaves and hooks (claws) and the biological activities of these extracts or purified compounds from them have shown anticonvulsive, anti-inflammatory, antimutagenic, antioxidant, cytoprotective, hypotensive and immunoregulatory effects [255-260]. This myriad of clinical indications might in effect be at least partially explained by different combinations of bioactive components compartmentalized into various plant parts and different *Uncaria* species (reviewed in [252]).

The chemistry of medicinal ingredients from plants

Regulatory concerns

Medicine as practiced today does not generally recognize any health benefits from natural products, and healthcare professionals that recommend nutritional supplements are motivated by their years of historical use in primitive societies. In addition, there is growing awareness that conventional medicines do not treat multi-factorial human health disorders such as anxiety, depression, chronic fatigue syndrome, arteriosclerosis, hypertension, pain or cancer successfully. The Physicians Desk Reference for Herbal Medicines (The physician's bible) does not list a single natural supplement that is approved for medical use. This is not because there are no efficacy data available, but because promising natural products have not been subjected to the strict regulation and the approval process that all pharmaceutical drugs need to have in order to be registered for clinical use (reviewed in [261]). However, nutritional supplements have survived the test of time, and in a lot of cases there is also scientific support of their efficacy (reviewed in [226]). The World Health Organization (WHO) estimate that 80 percent of the world's population, about four billion people, presently use herbal medicines for their primary healthcare, at a cost of billions of US dollars [262, 263]. These estimates even include developed countries such as Sweden [264]. Thus, herbal medicine is a major component in all societies industrially developed or not, and it is also a common element in the traditional practice of Ayurvedic, homeopathic, naturopathic, traditional oriental, and Native American Indian medicines.

One primary factor in the lack of regulatory approval of herbal products is the inability to define complex mixtures of bioactive components that exist in natural products sufficiently well enough to satisfy the good manufacturing practice (GMP) regulations imposed on pharmaceutical drugs. Despite this limitation several classes of active ingredients have been described, coming from a variety of sources from plant origin (See details in Medical plants and phytopharmaceuticals). In this

section of the thesis I describe those active ingredients of plant origin which have particular relevance to the immunologic modulating properties of *Uncaria tomentosa* extracts.

Current research development of plant extracts

The American Botanical Council (ABC) recently published a monograph entitled "The ABC Clinical Guide to Herbs" [265]. Here the most commonly used plant supplements are described together with all the available data published as of year 2001, supporting clinical indications, dosage/duration of use, chemistry, pharmacological actions, mechanisms of action, contraindications, adverse effects, drug interactions, regulatory status and clinical reviews of all reported studies. The most common clinical indication found for the plant extracts and formulations summarized in the ABC monograph was to treat immunologic/inflammatory disorders which in turn establish naturally occurring plant metabolites as an important source for identifying immune modulators for future pharmacological drug development. Moreover, the most common class of suspected active ingredients were simple organic acids although their contribution to overall efficacious health effects was not always clearly understood [265].

Basic plant chemistry

Description of the pharmacokinetics of plant bioactives is essentially impossible unless the actual chemical structures are known and can be quantified by high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) analyses. However, there are some general metabolic parameters that are well known and characterized. These are schematically shown in Figure 4. Essentially there are two known pathways that can lead to the synthesis of organic acids and other aromatized plant metabolites, the citric acid cycle and the shikimate acid pathway. The citric acid cycle is the result of aerobic oxidation of pyruvate via acetyl coenzyme A (CoA) to succinate for the necessary purpose of producing

Plant synthesis of bioactive ingredients, organic acids and aromatized efficacious components

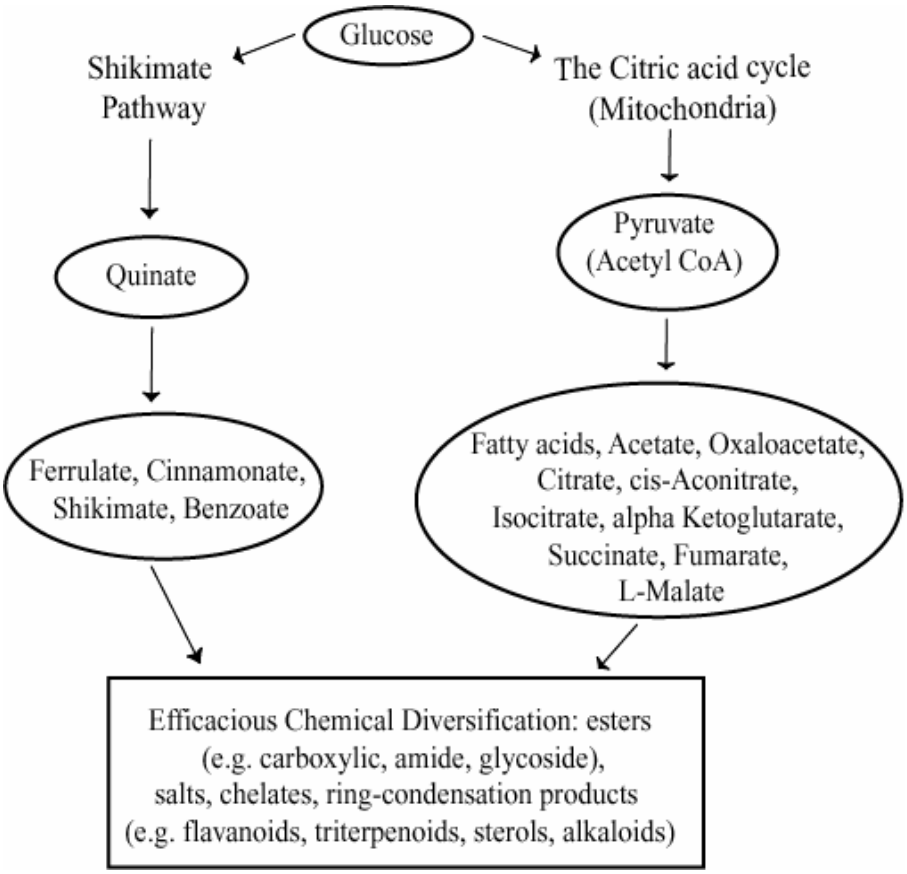

Figure 4. The known route of metabolism for plant production of organic acid salt forms and secondary aromatic metabolites.

energy (i.e. ATP/NAD). Other simple organic acids such as oxaloacetate, citrate, succinate, fumarate, or malate are by-products of this biochemical pathway. Fatty acids and other aromatized products are also either produced directly or modified by the citric acid cycle. Another pathway involving organic acids, which exists in

plants and micro-organisms, is the shikimate acid pathway. Here the key intermediate is quinic acid that is aromatized to benzoic acid via stepwise addition of three double bonds to the 6-membered ring of quinic acid yielding the intermediates cinnamonic acid, shikimate acid and then finally benzoic acid. According to this pathway aromatized ring-condensation products could be synthesized via the organic acid precursors already identified in the shikimate acid pathway [266, 267].

Chemistry of Uncaria tomentosa

The classes of active ingredients from *Uncaria tomentosa* products have been identified over the years since the early 1960's as oxindole alkaloids [268, 269], quinovic acid glycosides [256], novel polyhydroxylated triterpenes [255], steroids [270], procyanidins [271], and tannins [272]. To our knowledge all of the commercial preparations of Cat's claw except C-Med 100® or Activar AC-11® (a high dose form of C-Med 100®) are based on oxindole alkaloid content because of Dr Keplinger's discovery and structural elucidation of their presence in Cat's claw in 1967 [273]. However, Dr Pero and co-workers discovered in the 1990's that when Cat's claw extracts were prepared for human consumption according to the historical medicinal practice of the Asháninka Indians living in South America, there were only trace amounts of oxindole alkaloids present (i.e. <0.05%) [274]. These data were confirmed by other researchers preparing water soluble extracts of *Uncaria tomentosa* supporting the hypothesis that the oxindole alkaloid contents were not the primary active ingredients [275].

Since water soluble extracts of Cat's claw such as C-Med 100® did not have significant amounts of oxindole alkaloids present to explain it's activity, other already identified bioactive ingredients or previously unknown compounds were suspected to induce the biological effects. To further investigate this possibility C-Med 100® was chemically characterized as follows; (i) it had a UV absorption maximum of 200 nm, (ii) when subjected to activated charcoal adsorption, the

supernatant neither had UV absorption nor biological activity, (iii) the Bartos reaction for carboxy esters was positive but not after charcoal absorption. Based on these results it was concluded that the primary active ingredients of the *Uncaria tomentosa* water extract C-Med 100® were of the chemical class of carboxy alkyl esters (CAEs) because aromatic ring-substitutions would have shifted the UV spectrum away from 200 nm [274].

Chemical analysis of C-Med 100®

In order to carry out further chemical analysis C-Med 100® was dissolved in 90 percent ethanol and subjected to TLC [253]. The 90 percent ethanol solution was spotted on TLC silica gel 60 F254 plates, and then chromatograhed in a system of one percent ammonia in 95.5 percent ethanol. The TLC plate was scraped in 1 cm sections from baseline to solvent front, followed by elution of each section with one percent aqueous ammonia. Elution with aqueous ammonia proved to be necessary because of the very tight binding of the active ingredient to silica. Although the Rf = 0.3 spot was almost free from other Cat's claw components it contained relative large amounts of dissolved inorganic silica. In order to remove the inorganic component(s) introduced from the purification scheme off silica TLC plates, the one percent aqueous ammonia solution was freeze dried and the eluant was redissolved in methanol leaving behind the solubilized silica. Distilled water was added to the methanol eluant, then again freeze dried, before redissolving in water for bioassay of tumour cell growth inhibition using HL-60 cells as already described in Sheng et al. [276]. The only biological activity identified was located at Rf = 0.3 in the TLC chromatograms. The Rf = 0.3 compound had an ultraviolet absorption maximum at about 200 nm, absorbed onto charcoal, crystallized from methanol at -20° C and was further characterized chemically as CAE by the Bartos reaction with hydroxyl amine and ferric chloride [253, 277].

The crystallized active ingredient of C-Med 100® isolated by TLC was identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS) analyses to be

quinic acid [253]. However, quinic acid is not an ester but is easily converted to the ester quinic acid lactone by heat (Figure 5). In addition, it took strong base treatment of one percent ammonia to elute the active ingredient from silica gel. These chemical facts have strongly suggested that the identified quinic acid isolated from TLC could have been base hydrolyzed into quinic acid during isolation, and the natural occurring forms were quinic acid esters thus confirming the Bartos positive reaction of esters as the active ingredients in C-Med 100®. The natural occurrence of quinic acid alkyl esters as the active ingredients of C-Med 100® was finally resolved through a series of experiments. These established that; (i) quinic acid lactone as a model ester was susceptible to both acid and base hydrolysis demonstrating that one percent ammonia elution from TLC would form quinic acid, (ii) neither quinic acid lactone or quinic acid were bioactive unless treated with one percent ammonia, nor could they be identified as major components in C-Med 100®, (iii) when C-Med 100® was treated with strong base, the biological activity disappeared and quinic acid appeared as a product of the hydrolysis [253].

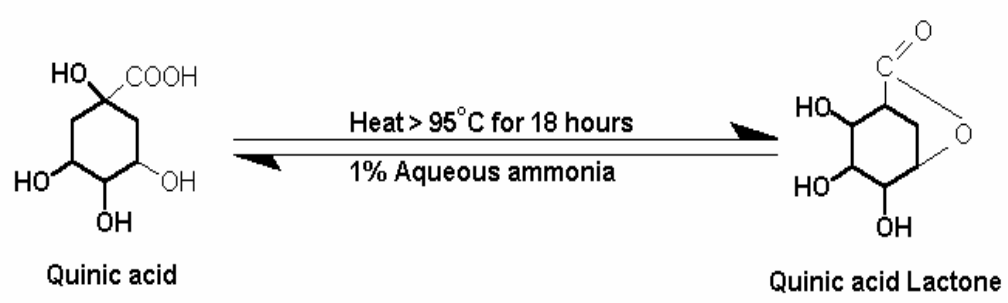

Figure 5. The chemical structures of quinic acid (QA) and quinic acid lactone (QAL).

Metabolism in the gastrointestinal tract

The digestive system

Digestion is a process that breaks down large molecules such as proteins, fats and carbohydrates into smaller molecules like amino acids, fatty acids and monosaccarides such as glucose, in order to facilitate systemic absorption into the body for support of the physiologic functions of life. This procedure is collectively performed by the digestive system which consists of the gastrointestinal tract (GI) and accessory digestive organs. The gastrointestinal tract includes the parts; mouth, pharynx, esophagus, stomach, small and large intestine. Accessory digestive organs are the liver, gallbladder and pancreas. In order to keep a metabolic balance in an organism six basic processes are performed; (i) intake of food and liquid, (ii) secretion of water, acid, buffers and enzymes, (iii) mixing and propulsion, (iv) digestion of food with the help of chemical and mechanical processes, (v) absorption of nutrients into the blood and lymph, and finally (vi) elimination of indigestible substances through defecation [278].

Food in the mouth is chewed and saliva containing α-amylase is added before entering the stomach where the most important enzyme is pepsin. Pepsin digests proteins into polypeptides and is most active at a pH of 2,0 to 3,0. An acidic environment is achieved by the gastric glands and the parietal cells that secrete a large quantity of hydrochloric acid.

Most of the digestion of carbohydrates, proteins and fats occur in the upper parts of the small intestine, in the duodenum and jejunum, with the help from the three accessory structures, pancreas, liver and gallbladder. The pancreatic juice contains a large quantity of α-amylase more powerful then that in saliva so almost all carbohydrates are digested to disaccharides before they have passed beyond the duodenum or upper jejunum. Furthermore proteolytic enzymes split proteins into small polypeptides and even individual amino acids. The last digestive stage of disaccharides and proteins are achieved by the enterocytes that line the villi in the

duodenum and jejunum. The enterocytes contain enzymes that are capable of splitting disaccharides to monosaccharides. In addition, the enterocytes have a brush border that consist of hundreds of microvilli that project from the surface of each cell. In the membrane of these microvilli multiple peptidases are contained and they split the remaining polypeptidases into tri- and di-peptides and further into amino acids [279].

Absorption by the organs in the gastrointestinal tract

The stomach is a poor absorptive area in the GI tract. Only a few highly lipid-soluble substances such as alcohol and some drugs like salicylic acid are absorbed in small quantities. About 90 percent of the absorption of nutrients and water take place in the small intestine by diffusion, osmosis and active transport. The other ten percents are absorbed in the large intestine [279]. The blood flow through the small intestine immediately recirculates into the liver by the way of the portal vein. In each region of the small intestine and in each layer of the gut wall this blood flow is directly related to the metabolic demands of the cells and their functional activity. During absorption the blood flow in the villi and adjacent regions of the submucosa increase by 30 to 130 percent of basal flow [280, 281].

Absorption of drugs in the gastrointestinal tract

The small intestine is regarded as an absorptive organ in the uptake of orally administrated drugs but it has also an ability to metabolize some drugs [282, 283]. Anatomically the small intestine is related to the liver concerning the absorption and the amount of orally administrated drugs that reaches the circulating blood might be reduced by both the intestinal and hepatic metabolism. The metabolism of drugs before entering the systemic circulation is called the first-pass metabolism and earlier it was widely believed that the liver was the major site of this metabolism because of its size and high content of drug-metabolizing enzymes (reviewed in [284]). However, recent clinical studies have indicated that the small

intestine contributes substantially to the first-pass metabolism of drugs like cyclosporine, nifedipine, midazolam and verapamil. Some studies have even suggested that the role of intestinal metabolism is greater than that of hepatic metabolism [285-289]. Many metabolic reactions occur in the gut wall and almost all of the drug-metabolizing enzymes that are present in the liver are also found in the small intestine. The cytochrome P-450 enzymes are involved in the biotransformation of drugs and foreign compounds and are homogeneous distributed in the liver but asymmetrically spread along the small intestine and the villi [290, 291].

Oral administration is the most widely used method for medication today in spite of all its disadvantages and shortcomings. Thus it is important to consider that the rate and extent of absorption can vary among individuals and even within the same individual (reviewed in [284]). Both biological and physiochemical factors might influence drug absorption. The biological factors include the gastric and intestinal transit time, membrane permeability, luminal pH, blood flow rate and first-pass metabolism. The physiochemical factors include the drug's intrinsic properties such as pKa, molecular size, lipophilicity and solubility [292, 293]. After oral administration the drug absorption occurs predominantly within the small intestine due to the large surface area which is provided by the epithelial folding and the villous structures. In humans, the mucosal surface area of the small intestine is approximately 200 m^2 and drug absorption across the gut wall can be mediated by either transcellular or paracellular transport or a combination of both. During transcellular transport drugs are transported through the epithelial cells into the blood while for paracellular transport drugs reach the blood via the tight junctions between epithelial cells. The contribution of transcellular and paracellular transport of drugs is dependent on the lipophilicity of the compound (reviewed in [284]).

Absorption of polyphenols in the gastrointestinal tract

Polyphenols are a group of molecules that are present in many plants, fruits, chocolate, tea, coffee, wine and fruit juices [294-296]. There are different classes of polyphenols and the main classes are flavonids and phenolic acids. One major source of the phenolic acids in plants are the metabolites of the shikimate pathway for example chlorogenic acid, the ester of caffeic acid and quinic acid that occurs in plant derived food products at high concentrations [296-298]. Chlorogenic acid and caffeic acid are both antioxidants and chlorogenic acid has been shown to inhibit DNA damage *in vitro* [299-303].

Oxygen-containing heterocyclic conjugates of phenolic acid flavanoids, are of considerable interest because of their proposed protective effects to atherosclerosis and certain cancers [304, 305].

Most flavanoids are glycosylated in food and this glycosylation influences the biological properties and the absorption through the gut wall [295, 297, 306]. The amount of intestinal absorption of chlorogenic acid is low and without its esterification with quinic acid, caffeic acid is absorbed more efficient [295]. In humans one third of the chlorogenic acid (33 %) and almost all of the caffeic acid (95 %) were absorbed in the small intestine [307].

Metabolism of C-Med 100®

C-Med 100® is prepared from the bark of *Uncaria tomentosa* as an ultrafiltrated hot water extract where high molecular compounds are removed [274, 308]. We suspected that most aromatic plant biosynthesis (i.e. containing double bonds and conjugated ring systems) of the chemical structures present in C-Med 100® probably were produced primarily by the shikimate pathway (reviewed in [309]). As a conformation of our interpretation one such biologically active compound that was found in C-Med 100® was quinic acid [253] and although QA in itself is not an

aromatic plant product it is the starting product in the shikimate pathway from which all other aromatic compounds are produced (Figure 4). The shikimate pathway does not exist in animals and metabolites synthesized via enzymatic conversion according to this plant pathway could not have been synthesized by animal biosynthetic routes. Therefore products coming from the shikimate pathway would be treated according to the natural catabolic routes existing in animals. This means that they would be eventually excreted as a xenobiotic, or be modified to products that could enter animal metabolic routes having nutritional or homeostatic value. Since the intestinal microflora has an operational shikimate pathway (reviewed in [309]) it is possible that the active ingredient in orally administrated C-Med 100® is modified in the GI tract before mediating health benefits. In this part of the thesis I discuss the shikimate metabolites as contributors to the effects of the C-Med 100® extract.

Administration of C-Med 100®

Several recent reports have demonstrated the efficacy of water soluble extracts of Cat's claw, administered to humans or animals by various methods such as; (i) oral administration by gavage, tablets or capsules [274, 308, 310, 311], (ii) oral administration in drinking water [197, 275, 312], (iii) *in vitro* exposure in cultures of cell lines and primary spleen cells [276, 313] and (iv) topical administration in human skin organogenic cultures *in vitro* [314]. The duration of administration in these studies varied from one day to eight weeks and yet they all were associated with efficacious effects. Collectively these data indicate that some of the active ingredients of C-Med 100® may not require GI tract microbial metabolism in order to mediate efficacy in animals or humans.

Identified metabolites of C-Med 100®

C-Med 100® is a multi-component crude extract and its pharmacokinetics can only be discussed in terms of identified bioactive components, since its metabolism probably is influenced by all ingredients that are present in the extract. Today the only known primary bioactive compound is a quinic acid analog (about four percent of the extract, see chemistry section and [253]).

Quinic acid exists mainly as an ester in C-Med 100® [253], but after oral administration it could be hydrolyzed to an active quinic acid form by the stomach pH. Furthermore it could become de-esterified by ubiquitous non-specific esterases, or synthesized to chlorogenic, ferrulic caffeic, hippuric or benzoic acids by the GI microflora. However, the shikimate pathway can be excluded as playing a significant metabolic role in the efficacy of quinic acid as a C-Med 100® active ingredient by the fact that topical application was quite efficacious, and yet skin has no capacity to generate the shikimate pathway metabolites. Likewise in animal cell cultures C-Med 100® extract showed various effects and here no shikimate pathway exist either thus, metabolites from this pathway can be eliminated as primary active ingredients (reviewed in [309]). Furthermore, the only catabolic activity of quinic acid that is known to exist in animals is its conversion first to benzoic acid by the microflora and then conjugation with glycine to hippuric acid, in the kidney and liver [315, 316]. Again neither skin or spleen cells is known to possess the enzymes to synthesize either benzoic acid or hippuric acid, thus eliminating the possibility that any of the shikimate metabolite was involved that could explain the efficacy of C-Med 100® in biochemical terms.

Nonetheless, after oral administration of quinic acid to rats 41.1 percent of the quinic acid dose ended up as hippuric acid after eight days in the blood plasma [317]. Thus, it became of scientific importance to establish that even though hippuric acid or for that matter other shikimate metabolites were not known to be produced in skin or splenocytes, they may already be present as active ingredients

in C-Med 100® at the time of application. In order to establish the lack of any shikimate metabolites in C-Med 100® we have used an UV absorption analysis.

Active ingredients of C-Med 100®

The active ingredient of C-Med 100® has been characterized as absorbing all the biological activity to activated charcoal. Furthermore, the only UV absorbing material removed by the charcoal had an UV absorption maximum of 200 nm. This indicate that any compound claming to be the bioactive ingredient of C-Med 100® should be absorbing UV at 200 nm [253]. Hippuric, ferrulic and isoferrulic acids all had UV absorption maxima between 227-323 nm and not at 200 nm, indicating that these metabolites are unlikely candidates to explain the *in vivo* efficacy of either C-Med 100® or quinic acid even if both animal or plant metabolism are taken into consideration [318]. Even though hippuric acid does increase in plasma over time after oral administration of quinate, and as such could influence the efficacy of orally administered C-Med 100® or quinic acid as a secondary metabolic mechanism, there is no scientific evidence that it is present in C-Med 100® or mediates the efficacy of the quinic acid esters as its active ingredients [253].

As described in paper III of this thesis, quinic acid is an active ingredient in itself *in vivo* and also *in vitro* if treated with one percent ammonia. However in C-Med 100® it appears to occur as quinic acid esters. There are several logical explanations for this apparent discrepancy which yet are unresolved and we will have to await the results of current studies actively being carried out at the present time. Possible explanations are that; (i) the quinic acid esters in C-Med 100® are hydrolysed to quinic acid by the low stomach pH generating the *in vivo* active quinic acid form, (ii) esterases in the GI tract convert quinic acid esters to quinic acid, the *in vivo* active quinic acid form (iii) quinic acid esters or quinic acid are both metabolized in the body to a new bioactive form such as a glycoside or glucuronide (iv) quinic acid

esters are broken down to quinic acid which in turn can form an endogenous stable chelate that in fact is the bioactive form. This alternative could explain the increased *in vitro* biological activity after ammonia treatment of quinic acid.

At any rate these metabolic possibilities which could explain how quinic acid is an important primary bioactive ingredient of *Uncaria tomentosa* water extracts, such as C-Med 100®, deserves to be further scientifically reviewed from a pharmacokinetic point of view.

This thesis

Background

The bark from the vine *Uncaria tomentosa* also called “Cat's claw” in English or “Ûña de gato” in Spanish, has been used for generations as an ”immunomodulator” by the Asháninka Indians in the Amazonas. Addition of bark to boiling water releases the active ingredients from the bark and the hot water is served as a tea to treat various disorders [272, 319]. Dietary supplements containing *Uncaria tomentosa* extracts are commercially available both in US and in Europe [273]. C-Med 100® also called Activare AC-11® is one such supplement. The preparation of this particular extract differs from many of the other products on the market, since C-Med 100® is derived through water extraction and removal of higher molecular weight components such as tannins and therefore only contains molecules with a molecular weight of less than 10 kDa [274, 308].

Previous reports have shown that C-Med 100® administered to experimental animals significantly accelerated the recovery from chemically induced leukopenia [274]. Further, C-Med 100® enhances both DNA repair and immune responses *in vivo* [308, 310]. These reported observations inspired us to explore the mechanisms of action of C-Med 100® and to attempt to identify the active components in the extract.

Aim of the thesis

The aim of this thesis was to identify the immunological basis for the beneficial effects of *Uncaria tomentosa* treatment on the immune system *in vivo*.

Our specific aims were:

(i) To investigate how C-Med 100® affects various cell populations in the immune system *in vivo*.

(ii) To analyse the impact of C-Med 100® on cell growth and its molecular mechanism of action.

(iii) To identify biologically active components in the C-Med 100® extract.

Paper I

"C-Med 100®, a hot water extract of *Uncaria tomentosa*, prolongs lymphocyte survival *in vivo*"

We were intrigued by the previously reported data on the impact of *Uncaria tomentosa* extracts on the immune system and began our studies in 1999. Thus to investigate the impact of C-Med 100® on the immune system of the mouse, C-Med 100® was supplemented at various concentrations in drinking water. This method was chosen to imitate the original use of the bark of *Uncaria tomentosa* as a remedy against various disorders. The effects on the immune cells were investigated at different time points during the treatment.

We observed that the number of cells in all leukocyte populations was proportionally increased in the spleens of the treated mice after a few weeks of supplementation. The increase in cell numbers were dose dependent and seemed to reach a steady state level after three weeks of supplementation, since continued treatment did only sustain the cell number at the same level. There was no accumulation of naïve, activated or memory cells in spleens from treated animals, indicating that C-Med 100® treatment did not select for particular cell subsets. Furthermore, histological evaluation of spleens from treated animals revealed no pathological changes. We also discovered that the increase in cell number was reversible and the number of cells returned to normal levels after four weeks following withdrawal of treatment. Previous studies had revealed an increase in peripheral white blood cell numbers in both treated rats and humans [308] thus the results in mice were in accordance with those earlier observations. We wanted to

understand how C-Med 100® treatment caused the accumulation of cells and came up with a number of mechanisms that could explain this phenomenon.

First, we investigated whether C-Med 100® might increase the production of naïve lymphocytes. We therefore studied the output rate of recent emigrants from the thymus to the periphery. Our results showed that there was no detectable increase in T lymphocyte production after C-Med 100® treatment. Furthermore, the subpopulations of precursor B cells in the bone marrow were similar in treated and untreated animals. These results suggested to us that C-Med 100® most likely did not influence the production or the out put rate of cells from the primary lymphoid organs. We decided therefore to look for other possible causes of the significant increase in spleen cell numbers in C-Med 100® treated animals.

Thus, we studied whether C-Med 100® treatment would influence cell survival and therefore the decay rate, since a decreased decay rate would be expected to increase cell numbers. To investigate this possibility we used an adoptive transfer system in which carboxyfluorescein diacetate succinimidyl ester (CFSE) stained splenocytes either from mice treated with C-Med 100® or from water supplemented mice were used as donor cells. These cells were transferred to recipients which had either been treated with C-Med 100® or only supplemented with normal tap water. We used recipients with full lymphocyte compartments, in which the donor cells would only proliferate slightly so that we would more accurately estimate decay rates. We determined the number of surviving, non-dividing CFSE positive cells in the spleen of the recipients at various time points after the transfer. The results showed that in untreated recipients, lymphocytes from C-Med 100® treated mice had a significantly lower decay rate as compared to cells from untreated animals. The C-Med 100® treated cells also decayed more slowly than untreated cells in the treated hosts. However, the difference was not as pronounced in these hosts. The reason for this difference might be that the untreated donor cells were influenced by C-Med 100® in their host and therefore decay more slowly. Further the steady state number

of lymphocytes in these recipients was higher because of the C-Med 100® treatment and therefore competition for resources and space might be more severe than in an untreated recipient animal. The cells transferred to a C-Med 100® treated recipient might therefore be expected to decay more rapidly. We concluded from these experiments that C-Med 100® prolongs the life time of treated cells and proposed that this is caused by anti-apoptotic components in the extract.

In this paper we also investigated the quality of the lymphocytes from treated mice and found that they proliferated equally well as cells from untreated animals after polyclonal stimulation *in vitro*. In addition, the treated and untreated cells went through the same number of divisions when assayed with CFSE-labelling. These results suggested that the accumulated cells were functionally normal. A previous report had shown that C-Med 100® treatment enhances DNA repair [308]. In addition, another *Uncaria tomentosa* extract containing also high molecular weight components, had been reported to protect cells against oxidative stress [197]. These are both properties that might prolong the life time of treated cells due to reduced apoptosis induction and perhaps also functionally explain our observation that cells accumulate due to a reduced decay rate.

Paper II

"An extract of *Uncaria tomentosa* inhibiting cell division and NF-κB activity without inducing cell death"

Uncaria tomentosa extracts have been reported to have several biologically interesting effects on cells *in vitro* [255, 256, 258, 260, 320]. Among those, extracts have been reported to induce proliferation arrest [321], apoptosis [276], inhibition of NF-κB activation [197] and inhibition of the production of the pro-inflammatory cytokine TNF-α [198]. We wanted to know the generality of these observations and

in particular whether the size selected C-Med 100® extract would be functionally similar to other extracts that also contain high molecular weight compounds.

We treated both cell lines and primary spleen cells with C-Med 100® extract *in vitro*. Cell proliferation, cell viability and induction of apoptosis were studied in the cultures. The results showed that C-Med 100® inhibits proliferation of tumour cells with varying efficiency and that it also inhibits mitogen-induced proliferation of normal mouse T and B cells. The observed inhibition of proliferation occurred at C-Med 100® concentrations where there was no toxicity or apoptosis induction in the treated cells. Actually we found that C-Med 100® treatment rather reduced apoptosis induction at concentrations that inhibited cell proliferation. These results support our finding in paper I that suggested that C-Med 100® contains anti-apoptotic compounds. Importantly, cell cycle analysis revealed no distinct cell cycle block in the treated cells even though proliferation was inhibited. Instead we observed that C-Med 100® treatment slowed down the division process, suggesting that the treatment rather retarded cell cycle progression without inducing a specific block. As mentioned above, a previous report showed that an *Uncaria tomentosa* extract also inhibited the activity of the transcription factor NF-κB [197]. Furthermore it had been published that a water derived *Uncaria tomentosa* extract inhibited the production of the inflammatory cytokine TNF-α in cells cultured *in vitro* [198]. These were intriguing results that first of all might explain the successful usage of *Uncaria tomentosa* extracts for treatment of inflammatory conditions but also explain the inhibition of cell proliferation observed in our experiments. Therefore the activity of the transcription factor NF-κB was investigated in C-Med 100® treated cells using two different biological assays. Our results showed that C-Med 100® dose dependently inhibited NF-κB activity in both assays, thus our results were in accordance with the previous study [197] and provided strong evidence that at least part of the NF-κB inhibitory activity of *Uncaria tomentosa* extracts was present in the low molecular weight fraction. We

also investigated if the observed inhibition of NF-κB activity might depend on a decrease in the degradation of the NF-κB inhibitory protein IκBα. However, our results showed no detectable difference in IκBα degradation. Thus the exact mechanism on how C-Med 100® inhibited NF-κB activity has to await further experiments.

Taken together, in this paper we showed that C-Med 100® inhibits proliferation of both cell lines and primary cells without inducing cell death. Furthermore C-Med 100® decreased the induction of apoptosis in primary cell cultures again suggesting that C-Med 100® had anti-apoptotic properties. Our results made us also speculate that the observed inhibition of proliferation may be partially caused by the inhibition of the NF-κB activity due to the central role of this transcription factor in cell growth and cell cycle control.

Paper III

"Quinic acid is a biologically active component of the *Uncaria tomentosa* extract C-Med 100®"

Our data in papers I and II, as well as previously published reports had identified several biological effects of *Uncaria tomentosa* extracts. Our main goal was to identify components in the C-Med 100® extract responsible for its biological effects. Therefore the chemical properties of C-Med 100® have been carefully analysed. Finally after a lot of work a biologically active component was isolated using thin layer chromatography. The isolated component was identified as quinic acid (QA), a substance that often occurs in plants and in plant derived food as an ester together with caffeic acid denoted chlorogenic acid [296-298] but also in free form in for example cranberries and sea buckthorn [322, 323]. After the identification, we began to investigate the efficacy of QA in the biological assays used in paper I and paper II. In these experiments we compared the biological activity of commercially available QA with the activity of the crude C-Med 100®

extract. The results showed that commercially available QA inhibited NF-κB activity in accordance with the previous findings using the crude C-Med 100® extract. However, while QA isolated from a fraction of the C-Med 100® extract inhibited the proliferation of the tumour cell line HL-60 cells, commercially available QA did not. One possible explanation for this discrepancy might be that the QA isolated from the C-Med 100® extract had been modified by the isolation process. In particular we suspected that the treatment of the material during size-selection by thin-layer chromatography with NH_3 might have caused that modification. To test this possibility, commercially available QA was treated with one percent NH_3 and lyophilized to dryness to keep it in the ammonium form. This ammonium salt form of QA was denoted QAA and was found to inhibit both cell proliferation and NF-κB activity. However, neither commercially available QA nor QAA had the capability to inhibit the induction of apoptosis in primary cell cultures as C-Med 100®. Commercially available QA also inhibited the break down of the IκBα protein, suggesting a plausible mechanism for the inhibition of NF-κB dependent reporter gene transcription. As already mentioned in paper II no such inhibition of IκBα protein break down was observed when we investigated the C-Med 100® extract in similar studies. Thus, these results together indicate that C-Med 100® and QA inhibit NF-κB activity by different mechanisms. One possible explanation for this observed discrepancy might be that QA is only one of the compounds in C-Med 100® that affects NF-κB signalling but other yet unidentified compounds with similar properties may also exist in this extract.

Most importantly, *in vivo* studies showed that after 21 days of treatment with QA in the drinking water led to significantly increased number of spleen cells similarly to treatment with the C-Med 100® extract. These results indicate that QA is one of the components that induced the accumulation of cells in the spleens of the C-Med 100® treated mice and strongly suggest that QA is an important bioactive component of the C-Med 100® extract. Taken together, starting from a phenomenon

of accumulation of leukocytes induced by treating mice with a complex extract (paper I), we have provided a likely mechanism for this accumulation (paper I) and in here finally isolated one component of the C-Med 100® extract that recapitulates the effect of the extract itself.

The next question to assess was whether all the biological activity of C-Med 100® could be accounted for by QA. HPLC analysis of the C-Med 100® extract revealed that only about two percent of the extract consisted of free QA [253]. Therefore we determined the presence of potential QA analogs in the C-Med 100® extract [253]. The results indicated that C-Med 100® contains about five percent QA ester analogs such as for example quinic acid lactone that can be formed from QA by heat. However the content of free QA and its analogs were not enough to account for all the biological activity of the C-Med 100® extract. It is possible therefore that the extract contains other thus far unidentified components with similar biological affects as QA. We have to date not been able to identify the ester analog forms of QA in the C-Med 100® extract. Furthermore QA could also be present in biologically potent salt or chelate forms. Future experiments will address these possibilities.

Populärvetenskaplig sammanfattning på svenska (summary in Swedish)

C-Med 100®, en naturmedicin med immunomodulerande egenskaper

Vårt immunsystem skyddar oss mot infektioner från bakterier, virus och parasiter. För att detta ska fungera behövs många olika egenskaper och idag delas immunsystemet in i två delar, en ospecifik och en specifik. Den ospecifika delen består av hud, slemhinnor, magsyra och olika celler som snabbt tar sig till ett område som blivit attackerat av någon mikroorganism.

Den specifika delen av immunförsvaret består bl.a. av B-celler och T-celler som samarbetar både med varandra och med andra vita blodkroppar. B-cellerna skapar en antikropp som är unik mot den mikroorganism som har invaderat och T cellerna dödar celler som har blivit angripna. Det tar lite längre tid att få igång en specifik respons men fördelen med denna är att den är speciellt riktad mot en invaderande patogen och att immunförsvaret kommer ihåg de mikroorganismer som har bekämpats eftersom minnesceller bildas. Minnescellerna kan sedan angripa samma organism snabbt och specifikt om den invaderar igen. Det är den här egenskapen hos vårt immunsystem som gör att vaccineringar mot olika sjukdomar fungerar och att vi vanligtvis inte får vissa infektioner som t.ex. röda hund, mässling, vattenkoppor mer än en gång i livet.

Även om immunförsvaret är ett effektivt försvarssystem behöver vi ibland använda mediciner när vi blir sjuka. Detta beror på att immunsystemet inte klarar av att rensa bort kraftiga infektioner eller komplexa sjukdomar som till exempel TBC, AIDS eller cancer. Dessutom finns det autoimmuna sjukdomar som uppkommer då de vita blodkropparna börjar anfalla kroppsegen vävnad. Det händer också att vi får missriktade immunsvar mot något som egentligen är ofarligt som vid olika typer av allergier.

För att behandla den här typen av tillstånd har människan ofta vänt sig till naturen och idag härstammar nästan tjugofem procent av våra mediciner från naturprodukter. Många av dessa, som till exempel acetylsalicylsyra i huvudvärkstabletter eller det smärtlindrande ämnet morfin upptäcktes redan tidigt och har använts i snart 200 år.

Klängväxten *Uncaria tomentosa*, växer i Amazonas regnskogar och har använts i generationer av lokalbefolkningen mot förkylningar och inflammationer. Barken har kokats i vatten så att de aktiva ingredienserna frigjorts och extraktet har druckits som te.

I den här avhandlingen har vi undersökt hur ett sådant extrakt påverkar immunförsvaret. C-Med 100® är ett vattenextrakt från *Uncaria tomentosa* som säljs som näringstillskott i USA. Tidigare undersökningar hade visat att C-Med 100® har en hämmande effekt på inflammatoriska tillstånd och en positiv effekt på återhämtningen av antalet vita blodkroppar efter cellgiftsbehandling. Dessa effekter är intressanta ur immunologisk synvinkel eftersom det behövs nya preparat mot inflammationssjukdomar och mot det minskade antalet vita blodkroppar som uppstår hos cancerpatienter efter cellgiftsbehandling. Det var därför viktigt att identifiera de mekanismer som låg till grund för hur C-Med 100® påverkade immuncellerna.

Våra studier har bland annat visat att om möss behandlas med C-Med 100® i dricksvattnet under tre veckor leder detta till en ökning av alla vita blodkroppar i mjälten. Cellökningen sker utan vävnadsförändringar och cellantalet återgår till normal nivå inom två veckor efter avslutad behandling. Dessutom har celltransplantationsförsök visat att C-Med 100® förlänger livslängden hos vita blodkroppar *in vivo*. Detta indikerar att C-Med 100® eventuellt innehåller komponenter som hämmar celldöden hos vita blodkroppar, en egenskap som kan vara användbar vid både leukopeni och andra sjukdomstillstånd.

Vidare har vi också observerat att C-Med 100® hämmar celldelningen (proliferation) hos både maligna celler och lymfocyter. Anledningen till detta är att

extraktet inhiberar aktiviteten av en viktig komponent som kontrollerar inflammationsresponsen, transkriptionsfaktorn NF-κB.

Eftersom de observerade effekterna är användbara vid behandling av sjukdomar är det viktigt att isolera den eller de aktiva substanserna i C-Med 100® extraktet. En kemisk komponent renades fram och den identifierades till att vara quinic acid (QA). QA har visat sig kunna öka antalet vita blodkroppar i mjälten efter tre veckors behandling i dricksvatten precis som C-Med 100® extraktet. QA inhiberar också aktiviteten av NF-κB. Dessutom har QA som behandlats med ammoniak och format ett ammonium salt (QAA) visat sig ha samma effekter som C-Med 100® extraktet.

I avhandlingen har QA identifierats som en aktiv substans i *Uncaria tomentosa* extraktet C-Med 100® och dessutom har våra studier ökat förståelsen för hur extrakten inhiberar inflammation. Vår förhoppning är att dessa resultat leder fram till utveckling av nya farmakologiska preparat antingen från QA eller från *Uncaria tomentosa* extraktet, som kan användas kliniskt.

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