Establishment and characterization of a murine T-cell lymphoma/leukemia model

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Cover image: Flax flower (*linum usitatissimum*). Flax seeds are rich in omega-3 fatty acids.©iStockphoto.com/constantgardener

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In memory of my mother and father

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Abstract

Mouse models of human disease are valuable tools for studying pathogenesis and for evaluating novel therapies. T-cell lymphoma is a relatively rare disease in humans, affecting 100-150 persons yearly in Sweden. It exists in both aggressive and more indolent forms. We have established a mouse model for an aggressive T-cell lymphoma, the T-cell lymphoma/leukemia (TLL) mouse. In the present thesis, the TLL mouse model was characterized and used for experimental therapeutic and primary prevention studies.

The TLL mouse was established unintentionally in our laboratory during work on V_H-gene replacement in a "knock-in" mouse experimental setting. The generated chimeras all developed aggressive T-cell lymphomas affecting the lymphoid organs, lungs, kidneys and liver. The lymphoma phenotype segregated from the targeted locus and we could demonstrate the presence of Moloney murine leukemia virus (MMLV) in germline of the affected mice. MMLV is a retrovirus known to induce T-cell lymphomas when inoculated in newborn mice. We further characterized two TLL substrains; TLL-2 and TLL-14 carrying the proviral integrations on chromosomes 2 and 14 respectively. Significant differences were found between the substrains regarding lymphoma frequency and immunophenotype, the TLL-14 substrain developing tumors with higher frequency than TLL-2 and with a more mature immunophenotype.

A transfer model was developed in which TLL cells could be readily transferred intravenously to syngenic recipients causing aggressive lymphomas. The transfer model was used in a therapeutic study where the selective COX-2 inhibitor celecoxib was evaluated as a single agent and in combination with the established anti-tumor agent cyclophosphamide. The study was based on results from other tumor types that have indicated celecoxib, originally an anti-inflammatory and analgetic drug, to have possible anti-tumor effects. In our TLL model, however, we could not demonstrate any benefit of celecoxib monotherapy or any additive effect to cyclophosphamide.

Dietary fatty acids, in particular omega-3 fatty acids, have been a focus of public and scientific interest due to observed effects on the prevention of cardiovascular disease, cancer and inflammatory conditions. In addition, omega-3 fatty acids inhibit T-cell proliferation in vitro. We supplemented the diet of TLL mice with omega-3 and omega-6 fatty acids respectively and could demonstrate a significant delay in lymphoma onset between 5-8 months of age in the group receiving an omega-3 rich diet.

Abbreviations

AA	Arachidonic acid
AICR	American Institute for Cancer Research
APC	Antigen presenting cell
A-TLL	Adult T-cell lymphoma/leukemia
CLL	Chronic lymphocytic leukemia
COX	Cyclooxygenase
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ES cell	Embryonic stem cell
FACS	Flow cytometry cell sorting
GEM	Genetically engineered mice
HL	Hodgkins lymphoma
HTLV-1	Human T-cell leukemia virus type 1
LOX	Lipooxygenase
LTR	Long terminal repeat
MCF	Mink cell focus
MHC	Major histocompatibility complex
MM	Multiple myeloma
MMLV	Moloney Murine Leukemia Virus
MUFA	Monounsaturated fatty acid
NHL	Non-Hodgkins lymphoma
NK cell	Natural killer cell
NKT cell	Natural killer T-cell
NSAID	Non-steroid anti-inflammatory drug
PAMP	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PG	Prostaglandin
PRR	Pattern recognition receptor
PTCL	Peripheral T-cell lymphoma
PUFA	Polyunsaturated fatty acid
RFLP	Restriction fragment length polymorphism
SCALE	Scandinavian lymphoma etiology study
T-ALL	T acute lymphoblastic leukemia
TCR	T-cell receptor
T-LBL	T lymphoblastic lymphoma
TLL	T-cell lymphoma/leukemia
TLR	Toll-like receptor
TX	Tromboxane
WCRF	World Cancer Research Fund
WHO	World Health Organization

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Eriksson B, **Johansson A-S**, Roos G, Levan G, Holmberg D (1999) Establishment and characterization of a mouse strain (TLL) that spontaneously develops T-cell lymphomas/leukemia. *Exp Hem*, 27:682-688

II **Johansson A-S**, Eriksson M, Norén-Nyström U, Larefalk Å, Eriksson B, Holmberg D (2006)
Germ Line Insertions of Moloney Murine Leukemia Virus in the TLL Mouse Causes Site-specific Differences in Lymphoma/Leukemia Frequency and Tumor Immunophenotype. *Anticancer research* 26:2873-2878

III **Johansson A-S**, Pawelzik S-C, Larfalk Å, Jakobsson P-J, Holmberg D, Lindskog M (2009)
Lymphoblastic T-cell lymphoma in mice is unaffected by Celecoxib as single agent or in combination with cyclophosphamide. *Leukemia & Lymphoma*;50(7):1198-1203

IV **Johansson A-S**, Norén-Nyström U, Larefalk Å, Holmberg D, Lindskog M
Fish oil delays lymphoma progression in the TLL mouse. Submitted for publication.

Populärvetenskaplig sammanfattning

Djurmodeller är viktiga för att förstå uppkomsten av många sjukdomar hos människor, och för att undersöka nya behandlingsmetoder. Särskilt musmodeller är användbara då möss och människor har många anatomiska och fysiologiska likheter, kombinerat med laboratoriemusens lättillgänglighet som modellsystem.
Lymfom är en tumörsjukdom som utgår från immunsystemets celler och som drabbar ca 1500 svenskar årligen. Lymfom delas in in Hodgkins lymfom och non-Hodgkinlymfom. De senare är vanligare (ca 90 % av alla lymfom) och kan i sin tur delas in i många undergrupper baserat huvudsakligen på vilken celltyp de har sitt ursprung i. 10-12% av non-Hodgkinlymfomen utgår från T-lymfocyter och det finns både aggressiva och snällare varianter av sjukdomen. Traditionellt behandlas de aggressiva formerna med cytostatika och i vissa fall strålbehandling, men resultaten är inte lika bra som för de typer av lymfom som utgår ifrån B-lymfocyter. Att undersöka nya behandlingar och metoder att förhindra uppkomsten av sjukdomen är därför värdefullt.

I den här avhandlingen har vi etablerat en ny musmodell för T-cellslymfom, TLL-musen, karakteriserat modellen och sedan använt den i en behandlingsstudie med en selektiv COX-2 hämmare, celecoxib samt i en preventionsstudie där vi analyserat olika fettsyrors inverkan på lymfomutvecklingen.

TLL-musen utvecklar spontant en aggressiv form av T-cellslymfom med engagemang framför allt i thymus (brässen), mjälte, lever och lymfkörtlar. Även benmärgen är engegerad av sjukdomen och därför kallas den lymfom/leukemi. Orsaken till sjukdomen är att ett så kallat retrovirus, ”Moloney murine leukemia virus” är infogat i DNAt i alla musens celler. Sjukdomen hos TLL musen liknar en sjukdom hos människor, adult T-cellsleukemi/lymfom, som beror på infektion med ett humant retrovirus, HTLV-1 och som är vanlig i vissa delar av världen (Japan, Karibien och Afrika). Vi har karakteriserat två stammar av TLL-musen som har viruset infogat på olika kromosomer. TLL-2 bär viruset på kromosom 2 och TLL-14 bär det på kromosom 14. Vi fann skillnader mellan stammarna i det att fler TLL-14 möss blir sjuka i lymfom (60% jämfört med 37% av TLL-2 mössen), lymfomet utvecklas i tidigare ålder (7 månader jämfört med 9 månader) och lymfomcellerna från TLL-14 mössen uttrycker proteiner på cellytan som tyder på att de utvecklats ur en mer mogen T cell jämfört med TLL-2.

Detta tyder på att platsen i DNAt där viruset finns, dvs den kromosomala lokalisationen påverkar hur viruset kan leda till lymfomsjukdom. Exakta mekanismen för detta är dock ännu oklar.

Om celler tas ur mjälten på en lymfomsjuk mus och injiceras intravenöst i en frisk mus utvecklas lymfom hos mottagarmusen efter ca 10 dagar. Detta sätt att använda TLL musen har fördelar framför att vänta på det spontana insjuknandet i lymfom. Dels blir alla injicerade möss sjuka och dels insjuknar mössen mycket tidigare, efter 10 dagar jämfört med efter flera månader. Vi har använt denna modell för att testa effekten av celecoxib, en selektiv COX-2 hämmare på T cellslymfom, något som inte gjorts tidigare. Celecoxib är ett registrerat läkemedel (Celebra®) som främst används mot värk, inflammation och feber, men det har också visat nytta i att förebygga vissa cancerformer, till exempel utvecklingen av tjocktarmscancer från polyper i tarmen hos personer med en ärftlig form av polypsjukdom. Vi gav celecoxib enbart, samt i kombination med cyklofosfamid, ett cytostatika som ofta används i lymfombehandling. I vår musmodell kunde vi inte se någon effekt av celecoxibbehandling, varken som enda behandling eller i kombination med cyklofosfamid.

Kostens betydelse för uppkomst av olika cancersjukdomar är ett ämne som intresserar både forskare och allmänhet. I djurstudier har man sett att rikligt intag av omega-3 fettsyrorna DHA och EPA som främst finns i fet fisk och fiskolja kan skydda mot uppkomst av vissa cancerformer och även öka effekten av cytostatikabehandling vid redan etablerad cancer. Denna positiva effekt av omega-3 fettsyror har inte kunnat bekräftas när man gjort stora studier i befolkningen, där forskare studerat sambandet mellan kost och utveckling av olika cancerformer. Dessa motstridiga resultat inspirerar till fortsatt forskning och vi har studerat effekten av att berika kosten med omega-3 och omega-6 fettsyror på utvecklingen av lymfom i vår TLL musmodell. Vi fann att hos möss som från 3 veckors ålder erhöll en diet berikad med fiskolja, dvs rik på omega-3 fettsyror fördröjdes insjuknandet i lymfom jämfört med möss som fick standardfoder. Skillnaden var statistiskt signifikant mellan 5-8 månaders ålder men försvann med tiden och vid 12 månaders ålder när studien avslutades sågs ingen skillnad mellan grupperna. Vi utförde en kompletterande studie med omega-3 och omega-6 berikat foder till friska möss för att se om olika dieter påverkade hur immunförsvarets olika celler utvecklades. Vi fann skillnader mellan dietgrupperna för vissa celltyper men om och hur detta bidrar till de observerade skillnaderna i lymfomutveckling är ännu oklart. Fortsatta studier behövs för att kartlägga detta.

Introduction

General introduction

This thesis focuses on T-cell lymphoma and modeling of this disease in mice. It describes a novel mouse model for aggressive T-cell lymphoma/leukemia and its use in therapeutic studies. We have focused on the influence of dietary fatty acids and an anti-inflammatory compound on lymphoma development and progression in our model. There is a close link between fatty acids and inflammation since fatty acids are precursors to a wide range of lipid mediators that regulate inflammatory and metabolic pathways. There is also much interest in dietary factors in general and lipids in particular, among cancer patients and cancer survivors, many of whom are spending considerable amounts of money on dietary supplements of doubtful efficacy. Every bit of knowledge adding to the field of cancer prevention and treatment, therefore, can be of value.

Overview of the immune system

The immune system protects the host from environmental pathogens such as bacteria, virus, fungi and parasites. It also permits tolerance to self-antigens and to non-threatening agents (e.g. food proteins). There is also evidence that the immune system regulates the development of cancer [1-3], but the tumor immune surveillance is not efficient enough to completely clear all potentially malignant cells from the body. Both cells and soluble factors are involved in the immune system. White blood cells (leukocytes), which constitute the cells of the immune system, are divided into lymphoid cells (T and B lymphocytes) and myeloid cells (granulocytes, monocytes and macrophages). The immune cells are produced from pluripotent hematopoietic stem cells in the bone marrow and then migrate to the lymphoid tissues throughout the body. The bone marrow and the thymus gland are considered primary lymphoid organs whereas spleen, lymph nodes and mucosa associated lymphoid tissue are the secondary lymphoid organs where immune responses are initiated. Generally, the immune system is divided into innate immunity and acquired/adaptive immunity[4].

Innate immunity

Innate immunity is the body's first line of defense against pathogens. The skin and the epithelial layers harbors dendritic cells, granulocytes, monocytes and macrophages that can recognize microorganisms based on the detection of conserved microbial components, known as pathogen-associated molecular patterns (PAMPs) [5]. These patterns are unique to the microbial world and often essential for the survival of the microorganism and therefore difficult for the microorganism to alter. The cells of the innate immune system recognize these patterns via pattern-recognition receptors (PRRs), and signal to the host the presence of an infection. There are several classes of PRRs, some of them expressed on the cell surface and some found exclusively in intracellular compartments where they are important in viral detection. The best characterized class of PRRs is the transmembrane toll-like receptors (TLRs). When recognizing a PAMP, the TLR trigger a multitude of anti-microbial and inflammatory responses. Most mammalian species have 10-15 different TLRs, with different speciality in what microbial products that they detect and what type of responses they trigger.

One specific feature of the innate immune response is the recruitment of inflammatory cells to the site of infection. This is mediated by TLR ligation that induce expression of chemokine and chemokine receptors that regulate cell migration. Circulating leukocytes are trapped by the vascular endothelium at the infection site and migrate from the blood stream into the tissue. TLRs also activate innate immune cells already present in the tissue as well as tissue stromal cells [6]. These recruited and activated cells of the innate immune system can destroy pathogens directly by phagocytosis or by the release of cytotoxic proteins.

Another important feature of the innate immune system is the activation of the body's second line of defence, the highly specific acquired or adaptive immune system. This activation is also mediated by the action of TLR signaling [7].

Acquired/adaptive immunity

The cells of the adaptive immune system are T and B lymphocytes, which allow for a highly specific and strong response to pathogens. During the development of T and B lymphocytes, a high level of diversity in antigen recognition is created by clonal selection. The system is also able to distinguish between non-self and self antigens so that normally it will only respond to foreign antigens. The B lymphocytes develop from precursors in the bone marrow to antibody producing plasma cells trough a tightly

regulated process. Non-reactive immature B cells migrate from the bone marrow primarily to the spleen. There they undergo at least two transitional stages before ending up as mature immuno-competent B cells ready for activation trough antigen receptor stimulation [8].

In normal T-cell development, T-cell progenitors are generated in the bone marrow and then migrate to the thymus gland. The T cells mature in the thymic cortex and T cells recognizing self antigens are eliminated in a process called negative selection. The different developmental stages can be recognized by the expression of certain cell surface molecules. Cortical thymocytes are initially double negative for the cell surface molecules CD4 and CD8. During the maturation process, the T-cell goes from the CD4/CD8 double negative stage to a double positive stage and finally expresses only CD4 or CD8. This differentiation is dependent on the expression of the pre-T-cell receptor (TCR). TCR gene rearrangement takes place in the double positive stage and a functioning T-cell receptor is formed. In the CD4 or CD8 single positive stage, the T-cell is considered mature and it migrates to the peripheral/secondary lymphoid tissue. T cells residing in the medullary thymus have the phenotype of mature T cells. CD4 positive T cells are called helper T cells and are subdivided into Th1 and Th2 cells based on their pattern of cytokine production. They provide help to other cells in the immune system; Th1 aid other T cells and macrophages whereas Th2 cells help the B cells in antibody production. CD8 positive T cells act as cytotoxic T cells with the ability to directly kill infected or otherwise targeted cells.

Antigen presenting cells (APCs) such as the dendritic cells and monocytes of the innate immune system activate the adaptive immune response by migrating from the infection site to the regional lymph node. There they present the captured microbial antigen to naïve T lymphocytes in the context of major histocompatibility complex (MHC) molecules. The naïve T-cell also need additional signals to become activated and these are induced by TLRs and include co-stimulatory signals and cytokines. In addition, TLRs plays a role in B-cell activation. Co-engagement of the B-cell receptor and TLRs enhance antibody responses to ensure a rapid response to re-infection. TLRs are also expressed on B-cells and stimulation by microbial antigen patterns can lead to polyclonal activation and production of IgM antibodies against common bacterial components in a T-cell independent manner [6].

During the immune response, signals delivered to B cells from T cells and *vice versa* are important and the tight collaboration between the cell types is initiated by antigen presentation on MHC class II molecules by B cells to T cells. Several types of specialized B and T cell subsets exist, all with specific tasks in the complex mechanism of adaptive immune response. One example

is the marginal zone B cells identified by the surface expression of IgM/CD20/CD21 and the absence of CD23 [9]. They reside in the marginal zone of the splenic white pulp or in the lymph node where they play an important role in transporting circulating immune complexes into the lymphoid follicles and depositing them on the surface of antigen presenting cells.

Other examples of specialized immune cells are the regulatory cells that suppress the immune response so that it is quantitatively and qualitatively adapted to give an optimal result. CD4+/CD25+ T cells [10-12], γδ T cells [13] and natural killer (NK) cells are all examples of regulatory cells [14]. One important effect of downregulation of immune responses by these specialized cells is protection from autoimmunity, i.e. when the immune system turns towards self-antigens leading to diseases such as diabetes, rheumatoid arthritis and inflammatory bowel disease [15-16]. The downside of immunosuppression could be less efficient defense against cells with malignant potential [17].

T cell lymphomas

Lymphoma is a malignancy of the immune system that affects more than 1500 Swedes every year [18]. Lymphomas can be divided into Hodgkins lymphoma (HL) and non-Hodgkins lymphoma (NHL). The more common NHLs (approx 90% of all lymphomas) can be further divided into B- and T-cell lymphomas depending on the cell type of origin. T-cell lymphomas are clonal tumors of immature or mature T lymphocytes at various stages of differentiation. They account for only 10-12% of all NHLs, the rest being of B-cell origin. The clinical presentation of lymphomas is often a swollen lymphnode in the neck, axilla or groin, but several other presentations are common such as abdominal or mediastinal masses or extranodal manifestations. Several patients have general symptoms at time of diagnosis such as weight loss, fever and sweating. Most of the patients with newly diagnosed lymphoma require immediate treatment, but some forms of lymphoma have an indolent course and treatment can be postponed for several years after diagnosis. Standard treatment for lymphomas in need of treatment is combination chemotherapy in repeated cycles. For B-cell lymphomas, the addition of an anti-CD20 antibody has improved outcome substantially and is now a part of the standard treatment. T-cell lymphomas are generally associated with inferior outcome than lymphomas of B-cell origin [19], and continuous effort is put into finding new treatment modalities or efficient combinations of existing treatments. Addition of an anti-CD52 antibody and high-dose chemotherapy with stem cell rescue are

currently being tested in clinical trials [20-21]. Radiation therapy is also important in lymphoma treatment, both with curative and palliative intention [22].

T-cell lymphomas resemble stages of normal T-cell differentiation and some of them can be classified according to the corresponding normal stage. However, for some of the lymphomas the normal counterpart is not yet identified [23]. In the 2008 World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissue, T-cell lymphomas are divided into T-acute lymphoblastic leukemia/lymphoma (T-ALL/LBL) originating from immature T cells in bone marrow or thymus, and mature T-cell lymphomas arising in peripheral lymphoid organs (Table 1).

WHO 2008 classification of T-cell neoplasms
Precursor T-cell neoplasms
Precursor T acute lymphoblastic leukemia/lymphoma (T-ALL/LBL)
Mature T cell neoplasms
Leukemic or disseminated
T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Adult T-cell lymphoma/leukemia
Systemic EBV-positive T-cell lymphoproliferative disorders of childhood
Extranodal
Extranodal NK/T-cell lymphoma, nasal type
Enteropathy-associated T-cell lymphoma
Hepatosplenic T-cell lymphoma
Extranodal cutaneous
Mycosis fungoides
Sezary syndrome
Primary cutaneous CD30+ lymphoproliferative disorders
Primary cutaneous anaplastic large cell lymphoma
Lymphomatoid papulosis
Subcutaneous panniculitis-like T-cell lymphoma
Primary cutaneous gamma-delta T-cell lymphoma
Primary cutaneous aggressive epidermotropic CD8+ cytotoxic T-cell lymphoma
Primary cutaneous small/medium CD4+ T-cell lymphoma
Nodal
Angioimmunoblastic T-cell lymphoma (AITL)
Anaplastic large cell lymphoma, ALK-positive
Anaplastic large-cell lymphoma, ALK-negative
Peripheral T-cell lymphoma, not otherwise specified (PTCL,NOS)

Table 1. WHO 2008 classification of lymphoid neoplasms.

T-acute lymphoblastic leukemia/lymphoma

T-ALL/LBL is, in the current lymphoma classification, considered as one disease as they share many common characteristics such as morphology and immunophenotype. The term lymphoblastic lymphoma is used when the neoplastic process is confined to a mass lesion. With extensive involvement of bone marrow and peripheral blood, the term lymphoblastic leukemia is appropriate. Having >25% blasts in bone marrow is often used as threshold for defining leukemia. Both T-LBL and T-ALL often present with a tumor mass in anterior mediastinum. Lymphadenopathy and hepatosplenomegaly are common. T-ALL and T-LBL can be stratified into pro-T and pre-T stages, cortical T stage and medullary T stage according to the surface markers expressed. Some studies suggest a correlation between the T-cell differentiation stages and clinical outcome [24-26]. T-ALL tends to show a more immature immunophenotype than T-LBL [27].

T-ALL comprises about 15% of ALL in children and 25% of ALL in adults. T-LBL, on the other hand, is far more common than lymphoblastic lymphoma of B cell origin, comprising about 85-90% of all LBLs.

Peripheral T-cell lymphomas

Peripheral or mature T-cell lymphomas are a heterogeneous group of T-cell neoplasms as indicated in Table 1. They are more frequent in Asia where they represent 15-20% of all non-Hodgkin lymphomas compared to 5-10% in the Western world. Peripheral T-cell lymphoma-NOS (not otherwise specified) is the most common subtype and accounts for approximately 30% of PTCL in the Western world. Adult T-cell leukemia/lymphoma (A-TLL) is a distinct subtype of PTCL with known etiology.

Adult T-cell leukemia/lymphoma

A-TLL is caused by the human retrovirus Human T-cell leukemia virus type 1 (HTLV-1). In 1970, a specific clinical entity was described in Japan – adult T-cell leukemia/lymphoma – and in 1979 the virus was isolated from a lymphoma patient [28]. The virus infection is endemic in several regions of the world like Japan, the Caribbean, South America, the Middle East and sub-Saharan Africa. Approximately 10-20 million people worldwide are infected. After a long latency period, 3-5% of infected persons develop A-TLL. The virus can also cause other types of disease, for example HTLV-1 associated myelopathy/tropical spastic paraparesis [29].

HTLV-1 can be transmitted in breast milk [30], through sexual contact [31] and through contaminated blood or blood products [32] and it causes a lifelong infection. The virus preferentially infects CD4 positive T cells but also CD8 positive cells [33] and it is transmitted as a cell-associated provirus, meaning that the infected T-cell hardly produces any virus and the plasma viral load is undetectable [34]. HTLV-1 does not carry a transforming oncogene. The oncogenic properties of the virus is instead mainly dependent on Tax, a viral transactivator protein that induces the expression of various host cellular genes involved in cell proliferation, survival and migration [35-37]. Also HTLV-1 bZIP factor is a viral protein that is of importance for leukemogenesis by promoting persistence of HTLV-1 infection and the proliferation of infected cells [38-39]. All neoplastic cells in A-TLL show monoclonal integration of HTLV-1.

Four clinical variants of A-TLL have been identified: acute, lymphomatous, chronic and smoldering [40]. The acute variant is most common and presents with elevated white blood cell count, lymphadenopathy, hepatosplenomagaly and often lytic bone lesions and hypercalcemia. In the majority of cases the malignant T cells express CD4 cell surface antigen and CD 25 is strongly expressed. In addition, tumor cells frequently express CCR4 and FOXP3 and therefore the postulated normal counterparts to the malignant cells are regulatory T cells [41].

The typical A-TLL patient responds poorly to conventional chemotherapy and has a median survival of less than one year for the acute variant.

Animal models for lymphoma/leukemia

Animal models in general, and especially mouse models, are irreplaceable tools for the study of carcinogenesis. The mouse shares anatomical, immunological and genomic similarities with humans and is the most accessible model system [42]. Hematological malignancies have been extensively studied in both inbred and outbred mice [43]. There are considerable differences between the many different laboratory mouse strains in terms of the type of disease they develop. Some of the variations are dependent on genetic factors and others are associated with the presence in the laboratory mouse genome of retrovirus-derived genes [44-45]. A goal in the studies of mouse leukemias and lymphomas is to identify the human counterpart to the studied disease in order to understand the pathogenesis of, and evaluate new treatments for, the disorder in humans. It is therefore important to classify the murine hematological malignancies and determine if the diseases in the two species are truly homologous.

Classification of mouse hematological malignancies

Human lymphoid malignancies are classified in the WHO 2008 classification according to clinical features, morphology, immunophenotype and genetic abnormalities [23]. A classification of mouse hematopoietic neoplasms was first published by Dunn in 1954 [46]. As for the case in humans, this classification has been revised several times. In 2002, the latest proposal (Bethesda classification) was published based on the 2001 WHO schemes for humans [47]. It is often difficult to determine the human homologue to a mouse hematologic malignancy (or vice versa!) and there are several reasons for this. Traditionally the different modalities used to classify human hematological malignancies have been used much less routinely for mouse tumors and also there are fundamental differences between humans and mice regarding the primary and secondary lymphoid organs. For example, extramedullary hematopoiesis continues in the splenic red pulp throughout the life of the mouse and the thymus persists to adulthood, in contrast to humans where hematopoiesis takes place in the bone marrow after the fetal period and the thymus is regressive after adolescence.

There is a broad range of lymphoid neoplasms seen in laboratory mice, occurring either spontaneously or after induction with radiation, chemicals or infection of newborn mice with leukemogenic viruses (Table 2). In addition to this, a large number of genetically engineered mouse (GEM) models exist, including transgenic mice, “knock-in” and “knock-out” mice, and tetracycline inducible systems. Some of these models were generated to model a specific human disease by introducing in the mouse the same genetic changes observed in the human neoplasm. Other types of leukemias and lymphomas seen in GEM have never been described in humans or as spontaneously occurring in mice [42, 47].

Bethesda 2002 classification for mouse lymphoid neoplasms
B cell neoplasms
Precursor B cell neoplasm
Precursor B-cell lymphoblastic lymphoma/leukemia (pre-B LBL)
Mature B-cell neoplasms
Small B-cell lymphoma (SBL)
Splenic marginal zone B-cell lymphoma (SMZL)
Follicular B-cell lymphoma (FBL)
Diffuse large B-cell lymphoma (DLBCL)
Morphologic variants
Centroblastic (CB)
Immunoblastic (IB)
Histiocyte associated (HA)
Subtypes
Primary mediastinal (thymic) diffuse large B-cell lymphoma (PM)
Classic Burkitt Lymphoma (BL)
Burkittlike lymphoma (BLL)
Plasma cell neoplasm
Plasmocytoma (PCT)
Extraosseous plasmocytoma (PCT-E)
Anaplastic plasmocytoma (PCT-A)
B-natural killer cell lymphoma (BNKL)
T-cell neoplasms
Precursor T-cell neoplasm
Precursor T-cell lymphoblastic lymphoma/leukemia (pre-T LBL)
Mature T-cell neoplasms
Small T-cell lymphoma (STL)
T-natural killer cell lymphoma (TNKL)
T-cell neoplasm, character undetermined
Large cell anaplastic lymphoma (TLCA)

Table 2. Proposed classification of mouse lymphoid neoplasms. Reprinted with permission from Blood journal. (Morse 2002).

Retroviruses

Retroviruses are a large and complex family of RNA-viruses. The hallmark of retroviruses is their ability to reversibly transcribe the virion RNA to double-stranded DNA followed by integration of this DNA into the genome of the infected cell. All retroviruses contain three major coding domains: *gag*, *pol* and *env*, which contain information for viral protein synthesis. When integrated in the host cell genome, the virus utilizes the cellular machinery for its replication [48].

Retroviruses were first associated with cancer about 100 years ago. In 1908 Ellerman and Bang, working in Copenhagen, isolated an avian erythroblastosis virus from a spontaneous erythroleukemia in a chicken [49]. In 1911, Rous in New York isolated a virus from a chicken sarcoma that

later was named Rous sarcoma virus [50]. These events were the beginning of oncoretrovirus research and tumor-inducing retroviruses were subsequently isolated from rodents, cats, cows, primates and other animals [51-52]. In 1979, the first human retrovirus was discovered, the HTLV-1 mentioned previously [28]. Retrovirus-induced malignancies of rodents provide informative model systems for elucidating the molecular mechanisms of carcinogenesis.

Retroviruses in general are considered benign, as the vast majority of them do not induce any pathology in the infected cell. When an infection is established, only a small amount of the cellular metabolism is committed to virus expression. An established retroviral infection can be suppressed by the host's immune response, but the virus is never fully eliminated and a chronic infection persists throughout life. Repeated infectious rounds occur and each infection event is associated with a proviral insertion that constitutes a mutation. Retroviral infection can, therefore, be thought of as similar to a massive exposure to a potent mutagen and eventually the insertions can alter the normal control of cell survival and division [53].

Retroviral oncogenesis

Retroviruses can induce malignancies in two major ways [48]:

1. By carrying cellular proto-oncogenes (acquired during recombination events between viral and host sequences) that, by integration in the host genome, can quite rapidly cause tumor formation. They are called acute transforming retroviruses and the prototype is Rous sarcoma virus that carries a transforming version of the *src* gene.
2. By insertional activation of host cellular proto-oncogenes. These viruses are called slow transforming because the neoplasm develops after a long latency.

For both the acute and slow transforming retroviruses, additional events are needed for the tumors to develop and tumorogenesis is considered a multistep process [54].

Leukemogenesis by Moloney murine leukemia virus

Moloney murine leukemia virus (MMLV) is a typical, slow transforming retrovirus that has been a useful model for the study of leukemogenesis. When inoculated in newborn mice, MMLV induces T-cell lymphoma with 100% incidence after a latency period of 3-4 months. The resulting lymphomas generally have the immunophenotype of immature T cells. The

leukemogenic process consists of multiple steps including proviral activation of cellular proto-oncogenes, generation of mink cell focus-inducing recombinants and virus induced changes in hematopoiesis [54]. Of these, insertional activation of proto-oncogenes is the most studied process [55-56]. The double-stranded viral DNA that is synthesized in the host cell cytoplasm after viral infection moves into the cell nucleus and integrates in the host genome. The place of integration is influenced by a number of factors including type of virus and characteristics of the target DNA such as methylation status and chromatin conformation. Several studies show preference for retroviral integration in actively transcribed DNA regions [57-59]. Most retroviral integrations do not alter the function of the host cell, but occasionally the provirus integrates in the vicinity of a proto-oncogene, which can result in transformation. The study of slow transforming retroviruses has led to the identification of many novel proto-oncogenes.

The provirus can lead to activation of adjacent genes through four mechanisms (Figure 1):

1. By action of strong viral promoters located in the downstream Long terminal repeat (LTR) of the virus directly on the proto-oncogene.
2. By action of the viral enhancer also located in the LTR of the virus on the promoter of the proto-oncogene.
3. By action of viral promoters in the upstream LTR of the virus leading to read-trough transcription of the provirus and into the downstream proto-oncogene.
4. By alteration of sequences that modulate expression of the proto-oncogene.

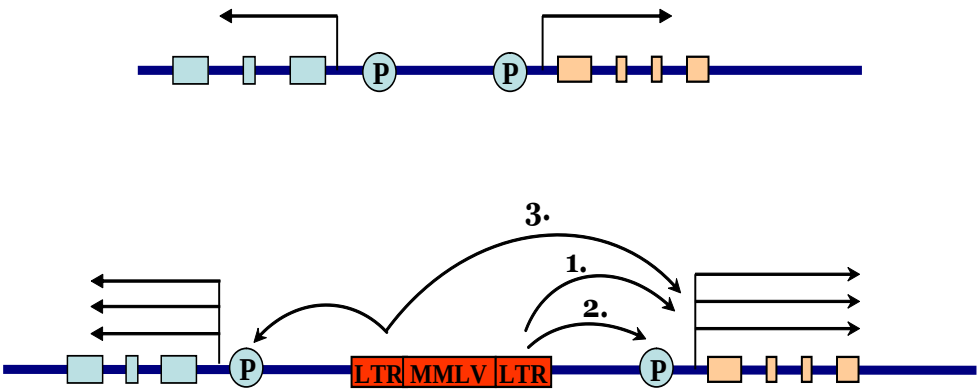

Figure 1. Top panel shows normal gene transcription initiated by promoters (P). Transcription can occur at both directions. Bottom panel shows that proviral insertion next to genes can lead to enhanced transcription by action of strong proviral promoters or enhancers in the LTR of the virus. Both 5´and 3´LTR regulatory elements can effect transcription.

A large number of common proviral insertion sites in tumors have been identified in retroviral tagging experiments [60-62]. The characterized gene products include transcription factors, growth factors, growth factor receptors, protein kinases and cyclins. In MMLV induced lymphomas, the genes most frequently affected by proviral insertions are c-*Myc*, which encodes a transcription factor that regulate approximately 10-15% of the genome, and *Pim-1*, which encodes a cytoplasmic protein kinase [63-66].

Another important step in the leukemogenic process is the formation of mink cell focus (MCF) viruses. These are *env* gene recombinants between the infecting murine leukemia virus (MLV) and endogenous viral sequences. It is known that any common laboratory mouse strain typically carries around 50-100 copies of endogenous MLV-related provirus, aquired during evolution of the strain [67-68]. These endogenous viral sequences are often transcriptionally silent, but occasionally RNA from these sequences is co-packaged into virions with exogenous virus and can recombine with the exogenous viral RNA. This can alter the host range of the virus by allowing interaction with other cell surface receptors than those utilized by the original virus, making the virus capable of additional rounds of infection. Additional rounds of infection mean more opportunities for the virus to integrate near sensitive genes and thereby potentiating leukemogenesis.

Events other than proto-oncogene activation and the appearance of MCF viruses are also important for neoplastic transformation. Viral infection of precursors to thymocytes in the bone marrow or spleen can lead to inhibition of hematopoiesis in the bone marrow and a compensatory hyperplasia of the spleen [69]. Hyperplastic lymphoid progenitors migrating from spleen to the thymus gland where additional leukemic events take place could result in a T-cell lymphoma [70].

Omega-3 and Omega-6 fatty acids

Fatty acids are heterogeneous groups of hydrophobic molecules composed of hydrocarbon chains with a carboxyl group on one end and a methyl group on the other. The length of the carbon chain and the type of bond between the carbons divide the fatty acids into different groups with distinct properties. Fatty acids containing only single carbon-carbon bonds are referred to as saturated. When one double bond between carbons is present, the fatty acid is called a mono-unsaturated fatty acid (MUFA) and when the chain contains more than one double bond it is called a poly-unsaturated fatty acid (PUFA). PUFAs can be subdivided based on the position of the first double bond in the hydrocarbon chain such that omega-3 PUFAs have the first double bond 3 carbons from the methyl end and omega-6 PUFAs have the double bond 6 carbons from the methyl end (Figure 2.)

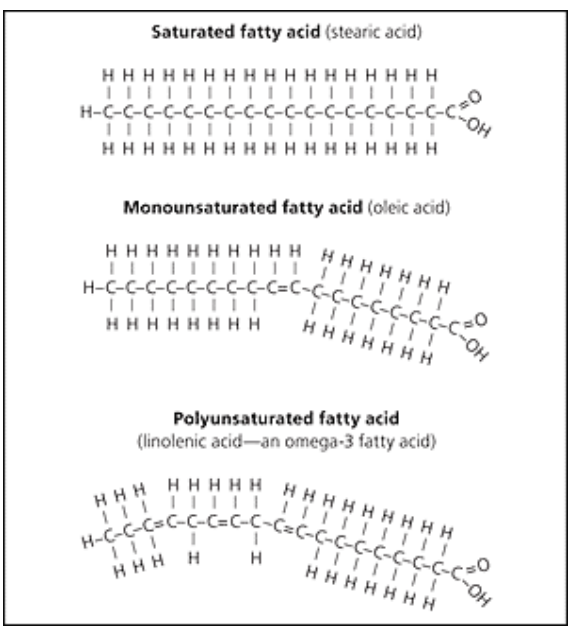

Figure 2. Molecular structure of fatty acids. Reprinted with permission from American Family Physician (White 2009).

Fatty acids are the main components of dietary fats and are major substrates for energy production in the body as well as an important compound of cellular membranes. Mammals can synthesize saturated and monounsaturated omega-9 fatty acids but cannot synthesize the omega-3 or omega-6 double bond. Thus, these fatty acids are essential and must be obtained trough the diet. Omega-6 fatty acids are consumed mainly as linoleic acid from most vegetable oils including sunflower and corn oils. Omega-3 fatty acids can be consumed as α-linoleic acid from flaxseed oil, canola oil and soybean oil but also as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are found in fatty fish and fish oils. Humans have the enzymes to convert α-linoleic acid to EPA and DHA but the pathways are not effective and the best source of DHA and EPA is a diet rich in fatty fish or fish oils [71].

Omega-3/omega-6 in inflammation and immune response

Fatty acids play several different roles in the cells of the immune system [72-74]. They serve as source for energy, they contribute to the functional properties of cellular membranes, they directly regulate expression of some genes and, importantly, they serve as precursors for biosynthesis of bioactive lipid mediators, eicosanoids and docosanoids (described in detail below).

The cellular membrane is a barrier between the cellular structure and the extracellular milieu, and is a structure with many properties. The omega-6 long chain fatty acid arachidonic acid comprises about 15-20% of the typical immune cell membrane in both rodents and humans while very little long-chain omega-3 fatty acids (EPA and DHA) are found in these membranes [75-76]. Dietary intake of different fatty acids affects the membrane phospholipid composition and can influence immune cell function by alterating membrane order and raft function [77-79]. Lipid rafts are organized membrane microdomains that play an important role in localization and distribution of receptors and signaling molecules. Altering phospholipid composition of membrane rafts by adding omega-3 PUFAs has been shown to suppress signal transduction in T cells [80-81]. Altered composition of the membrane can also affect the phagocytic capacity of the immune cells with phagocytic properties [75].

EPA and DHA can also directly inhibit the production of inflammatory cytokines in cell cultures [82-84] as well as in humans *in vivo* [85-86]. These effects seem to be due to altered expression of cytokine encoding genes by modifying transcription factors such as nuclear factor κB and PPAR-γ [87-88]. The immunosuppressive effects of omega-3 fatty acids are thought to be beneficial in some inflammatory related diseases such as asthma,

inflammatory bowel disease and rheumatoid arthritis [89-91]. On the other hand, several animal studies have shown deteriorated defense against viral infections after omega-3 dietary supplementation [92-93].

Omega-3/Omega-6 in cancer

In several animal studies of various types of cancers, it has been shown that dietary supplementation with omega-3 fatty acids slows tumor growth. Increased efficacy of chemotherapy and radiotherapy has also been proposed upon increasing the amount of omega-3 fatty acids in the diets of treated animals [94]. Several of the tumor inhibiting effects can be exerted trough the same pathways as the immunomodulatory effects previously described. Omega-3 fatty acids downregulate activation of oncogenic transcription factors as well as inflammation-promoting factors, many of which promote tumorigenesis [95-96]. In addition, omega-3 fatty acids have been shown to induce differentiation in certain tumor cells [97] and also to inhibit angiogenesis through multiple mechanisms [98-99].

As opposed to the positive effects of adding omega-3 to experimental diets, the supplementation of omega-6 has been shown to promote tumor growth in animal studies [100-101]. A proposed mechanism is enhanced production of AA with the subsequent formation of tumor-promoting prostaglandins. Studies on omega-6 fatty acid are, however, not as abundant as studies of omega-3 fatty acid and the role of this class of fatty acid in cancer development is not clear.

Dietary fat in cancer epidemiology

The relationship between dietary fat intake and cancer has been extensively studied for more than 20 years. The debate is still ongoing despite numerous populations-based and case-control studies; however, there are several reasons why conclusions are difficult to make. One crucial question is whether fatty acids play a part in cancer development beside their contribution to cellular energy production. It is known that obesity is a major risk factor for cancer [102] and a meta-analysis of 14 studies on laboratory mice found that energy restriction resulted in 55% reduction in spontaneous mammary tumors [103]. The international literature on food in cancer prevention was reviewed in a large report by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR). In this extensive work, the role of specific fatty acids was not covered due to insufficient data [104] but other reviewers have looked into the specific role of omega-3 fatty acids on cancer risk [105-106]. In MacLean's review, 38 studies on 20 prospective cohorts were included

totaling over 700 000 participants with observation periods ranging from 3-30 years. Eleven different types of cancer were described with more than half concerning breast, colorectal and prostate cancer. Six different ways to assess omega-3 intake using dietary questionnaires were used with fish consumption as proxy for EPA and DHA consumption used in many studies. In summary, only 10 out of 65 estimates of association were statistically significant and there were significant associations for both increased and decreased risk for cancer with high omega-3 intake.

The WCFR/AICR international group has published 2 reports, in 1997 and 2007, on food, nutrition and the prevention of cancer. Gerber reviewed these data along with studies published between 2006 and 2008 [105]. The WCFR/AICR report of 2007 noted limited evidence that eating fish protects against colorectal cancer in contrast to animal data where several investigators observe inhibition of colon tumorigenesis by omega-3 rich diets. Addition of more recent studies conclude that fish intake probably decreases colorectal cancer risk, but only limited evidence exists for a relationship between omega-3 fatty acid intake and cancer risk reduction. For prostate cancer, one study reported risk reduction associated with high intake of fatty fish, but two studies reported no risk reduction. In the case of breast cancer, limited but suggestive evidence exists that high consumption of fish and omega-3 fatty acids is associated with reduced breast cancer risk.

The apparent conflict between the promising animal data on omega-3 intake and cancer risk reduction and the weak associations reported in human dietary studies is interesting. Does it mirror the fact that animal models are far from replicating human conditions or is it a matter of dose? The high doses of omega-3 fatty acids supplemented in many animal studies are hard to achieve in humans and could also give substantial side effects such as coagulopathy.

In the meta-analyses and reports described above no significant associations were found between omega-6 intake and risk for cancer, however some studies reported a higher risk for certain types of cancer with a diet high in polyunsaturated fat. In a modern, Western diet the ratio between omega-6/omega-3 PUFAs is 15-20, meaning that a diet rich in PUFAs is likely to be rich in omega-6.

Dietary fat in hematological malignancies

The Scandinavian Lymphoma Etiology (SCALE) study is a large, population-based, case-control study of newly diagnosed lymphoma patients and controls in Sweden and Denmark. The authors could show that high dietary intake of marine fatty acids was associated with significantly lower risk for NHL [107]. Dietary intake of omega-6 was not associated with altered risk for NHL except for an inverse association with follicular lymphoma. A large, Canadian case-control study of patients with leukemia, multiple myeloma and NHL [108] found that increased weekly servings of fresh fish had a protective effect on all three malignancies. Other studies, i.e. The Nurses Health Study and Iowa Women's Health Study, failed to prove a significant association between fish/marine omega-3 consumption and incidence of NHL [109-110].

A study on dogs with lymphoma [111] showed increased survival in the treatment group fed an omega-3 supplemented diet in addition to chemotherapy. In this study the omega-3 rich diet correlated with increased serum levels of DHA and EPA. In contrast to this and several other animal studies consistently showing anti-tumoral effects of omega-3 fatty acids, a recently published report showed that dietary omega-3 enhanced the metastatic potential of murine T-lymphoma cells transplanted into B6 mice [112].

Eicosanoids and docosanoids

Eicosanoids and docosanoids are signaling molecules produced by oxidation of 20- or 22-carbon fatty acids (*eicosa* being the Greek word for twenty and *docosa* for twenty-two). They regulate several processes in the body, mainly in inflammation and immunity, but also act as messengers in the central nervous system. Eicosanoids are derived from either omega-3 or omega-6 fatty acids. There are four families of eicosanoids: prostaglandins, prostacyclins, thromboxanes and leukotrienes. Each of these exists in two or three separate series depending on whether they are derived from omega-3 or omega-6 fatty acids (Figure 3). The omega-6 derived eicosanoids are generally pro-inflammatory and the omega-3 derived eicosanoids have anti-inflammatory properties, but exceptions to this generalization exist. The balance of omega-6 and omega-3 fats in the diet will affect the eicosanoid-controlled funtions in the body. Docosanoids are mainly produced by oxidation of docosahexonic acid (DHA) and include resolvins, signaling molecules important in resolution of inflammation, and docosatrienes [113].

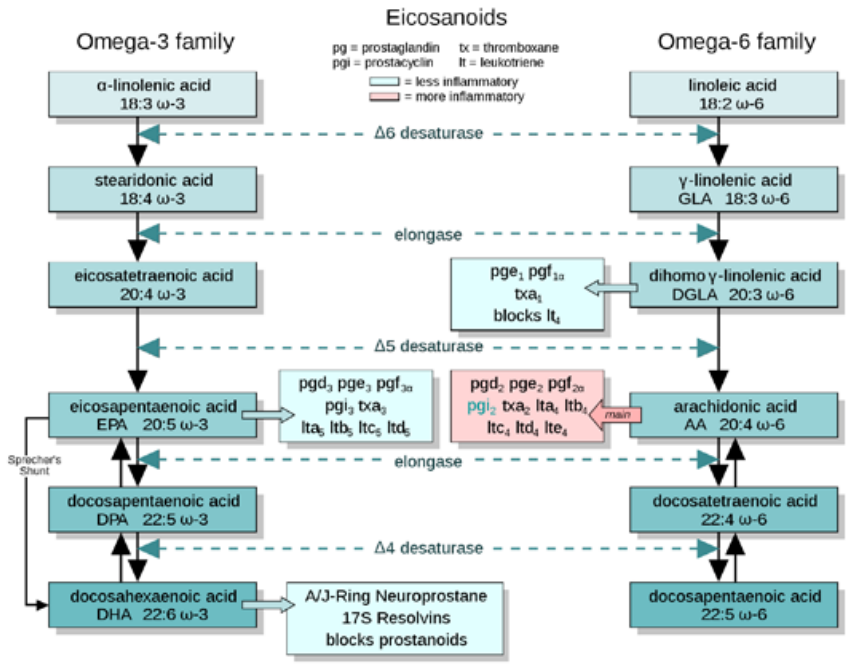

Figure 3 Essential fatty acid production and metabolism to form eicosanoids. At each step, the omega-3 and omega-6 cascades compete for the enzymes. Figure by David R Throop.

Arachidonic acid (an omega-6 PUFA) is the most abundant essential fatty acid in the cellular membrane and after release from the membrane by phospholipase A2 it acts as a precursor in the arachidonic acid cascade. Three families of enzymes catalyze the production of eicosanoids from arachidonic acid: cylooxygenases (COX), lipooxygenases (LOX) and cytochrome P450 isoforms . Examples of eicosanoid actions in the body besides their immunomodulatory functions are mediation of pain and heat sensations, promotion of sleep, protection of the gastric mucosa protection, contractions of the uterus and aggregation of platelets [114-115].

Cyclooxygenase enzymes

Cyclooxygenases (COX) catalyze the biosynthesis of prostanoids (prostaglandins and thromboxane A_2) from arachidonic acid (Figure 4). Two isoforms of COX exist in mammals, the constitutively expressed COX-1 and the inducible COX-2 [116]. COX-1 function includes gastric and renal protection and platelet function. COX-2 is induced in different cell types in response to proinflammatory stimuli and is responsible for prostaglandin

production during inflammatory responses, reproduction and systemic stress. The COX isoforms have been demonstrated to play distinct roles in T-cell development. COX-1 is expressed in maturing thymocytes and defiency of COX-1 results in early blocking of transition of T lymphocytes from the double-negative to the double-positive stage. COX-2 inhibition selectively affects the maturation of CD4 single-positive thymocytes [117].

Prostaglandins have an immunoregulatory role affecting both T and B lymphocytes. PGE_2, one of the most well-studies prostaglandins, suppresses T-cell activation and proliferation trough different mechanisms [118-120] and it also inhibits B-cell activation, proliferation and differentiation [121-122].

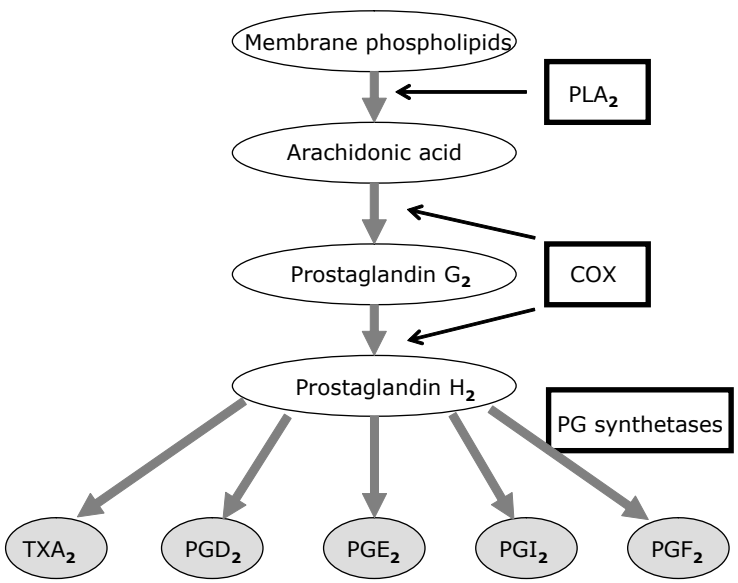

Figure 4. Arachidonic acid metabolism. Phospholipase A_2 (PLA_2) converts membrane phospholipids to arachidonic acid, which is the substrate for the cyclooxygenase enzymes COX-1 and COX-2. PGG_2 is reduced to PGH_2 which is then converted by specific cellular synthetases to a biologically active product.

COX-2 in cancer

Overexpression of COX-2 has been demonstrated in several tumor forms including colon cancer, breast cancer and lung cancer [123] as well as in premalignant lesions such as Barret´s esophagus, leukoplakias of squamous epithelium and ductal carcinoma *in situ* of the breast [124]. Overexpression

of COX-2 leads to increased levels of prostaglandins in tumors. Prostaglandins affect multiple pathways important in carcinogenesis including stimulation of angiogenesis, increased resistance to apoptosis and increased tumor cell invasiveness [125-128]. Prostaglandins act by binding to specific G-protein coupled receptors and thereby activate several intracellular signal transduction pathways. Multiple prostanoid receptors have been cloned and the PGE2 receptors EP1-4 play a significant role in the development and growth of tumors as demonstrated by suppression of carcinogenesis in EP receptor knockout mice. COX-2 knockout mice also show a reduction in development of intestinal tumors and skin lesions [129-130]. Numerous pharmacological studies using COX-2 inhibitors have shown reduced formation of several tumor types both in experimental animals and in humans.

COX-2 in hematological malignancies

In recent years, the interest in COX-2 in cells of hematopoietic origin has increased, supported by the findings that prostaglandins have multiple effects on B- and T-cell development. Normal, resting B cells do not express COX-2 but activated B cells, as well as chronic lymphocytic leukemia (CLL) B cells, show high levels of COX-2-protein [131-133]. Elevated expression of COX-2 has also been described in Burkitt´s lymphoma cell lines [134].

Increased COX-2 expression has been detected *in vivo* in many types of hematological malignancies including CLL, multiple myeloma (MM) and NHL [135-137]. This increased expression is often an indicator of poor prognosis. In a study by Paydas and coworkers, overexpression of COX-2 as determined by immunohistochemistry was detected in 56% of NHLs (177 cases) with association to aggressive histology but not to response to chemotherapy [138]. Another study of lymphoma conducted by Hazaar and coworkers found COX-2 expression in 57% of NHL cases (42 patients studied) and 70% of HL cases (10 patients studied). In this study, COX-2 expression correlated with advanced stage and poor response to chemotherapy [139]. One of few studies on COX-2 in T-cell lymphomas found immunohistochemical positivity for COX-2 in 71% of patients with NK/T-cell lymphoma, nasal type (34 patients) and the authors showed that COX-2 expression significantly correlated with shorter disease free survival and overall survival [140].

Non-steroid anti-inflammatory drugs

Non-steroid anti-inflammatory drugs (NSAIDs) are among the most commonly used medications worldwide. They are primarily used for their anti-inflammatory, analgetic and anti-pyretic properties but also, in the case of aspirin, for prevention of cardiovascular disease [141]. In more recent years, they also have been shown to have preventive effects on colorectal and breast cancer and on other malignancies [142-144]. The older generation of NSAIDs acts by blocking both COX-1 and COX-2 and thus inhibits the production of prostaglandins and thromboxanes [145]. The main side effect of this treatment arises from blocking the gastroprotective effects of COX-1 induced prostaglandins resulting in gastritis and gastric ulcers. The selective COX-2 inhibitors, including celecoxib, rofecoxib, valdecoxib, etoricoxib and lumiracoxib, were developed and initially showed markedly reduced side effects. However, as they became widely used an increased risk of cardiovascular disease was clearly associated with long-term use [146]. This resulted in withdrawal of rofecoxib and valdecoxib from the market in 2004 and 2005 respectively. Celecoxib has not been associated with cardiovascular side effects to the same extent and is still on the market. Over 3000 studies have investigated the effects of selective COX-2 inhibitors since they were introduced in late 1990s and several of these studies have focused on their anticarcinogenic potential [147].

Celecoxib in cancer

When it comes to the use of selective COX-2 inhibitors for cancer prevention and treatment, celecoxib was first approved by US Food and Drug Administration for preventing the formation of pre-cancerous intestinal polyps in patients with familial adenomatous polyposis [148]. As of today's date, 160 completed and ongoing clinical trials of celecoxib in the prevention and treatment of cancer can be found at the National Institute of Health (NIH) web page www.clinicaltrials.gov. Several of the trials concern the addition of celecoxib to chemotherapy or radiotherapy for different types of cancer and others concern cancer prevention in certain risk groups. In several animal models of epithelial cancers, COX-2 inhibitors have been shown to markedly decrease tumor growth and metastasis [149-153].

Antitumor effects of selective COX-2 inhibitors have also been reported for hematological malignancies. *In vitro* studies showed reduced viability of B-CLL cells after treatment with both non-selective COX-1/COX-2 inhibitors and selective COX-2 inhibitors [135]. Celecoxib inhibited proliferation and induced apoptosis in Burkitt´s lymphoma cell lines [154]. A clinical study of

heavily pretreated patients with relapsed/refractory NHL showed a 37% response rate to the combination of low-dose “metronomic” cyclophosphamide in combination with high-dose celecoxib. The authors suggest anti-angiogenic mechanisms for the observed responses [155].

The observed antitumorigenic effects of celecoxib were first thought to be solely mediated by blocking the COX-2 enzyme leading to a decrease in prostaglandin production. The inhibition of COX-2 has undisputedly clinically relevant antineoplastic effects, but over time additional drug targets have been discovered that do not require the presence of COX-2 to exert their activities (Figure 5). Direct targets of celecoxib apart from COX-2 are protein dependent kinase-1 (PDK-1), cyclin dependent kinases (CDK´s), sarcoplasmic/endoplasmic reticulum (ER) calcium ATPase (SERCA) and carbonic anhydrase (CA), which are main components in various cellular pathways. Generally, inhibition of these proteins by celecoxib leads to induction of apoptosis, inhibition of cell cycle progression, angiogenesis and metastasis. The entire range of molecular mechanisms of celecoxib activity, however, is still not known [147] [156].

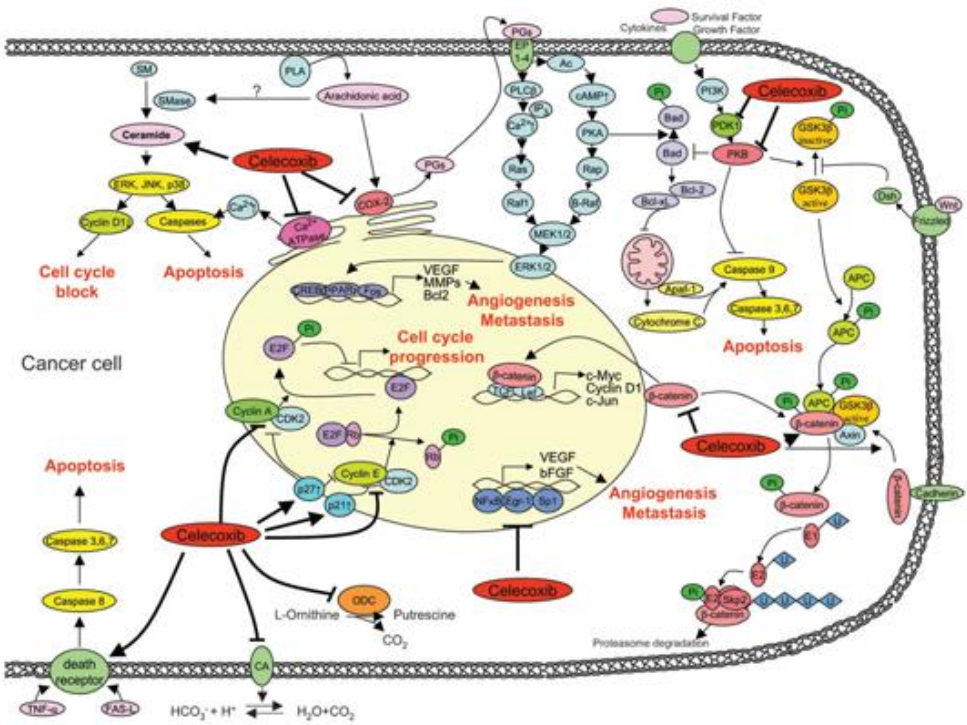

Figure 5. Molecular mechanisms of celecoxib and its anticarcinogenic effects. Reprinted with permission from J Natl Cancer Inst and the author. (S Grösch, 2006).

The antitumorigenic potency of celecoxib seems to be unique among selective COX-2 inhibitors probably due to the COX-2 independent drug targets. Structural analogues of celecoxib lacking the COX-2 inhibitory properties can mimick the anticarcinogenic effects of celecoxib and they may represent a new class of drugs for cancer prevention and treatment [157-159].

Aims

- To characterize the newly established T-cell lymphoma/leukemia (TLL) mouse strain.

- To understand the role of Moloney murine leukemia virus (MMLV) in lymphomagenesis in the TLL mouse strain.

- To investigate the effect of the selective COX-2 inhibitor celecoxib on T-cell lymphoma/leukemia originating from TLL mice.

- To investigate the effect of dietary supplement with omega-3 and omega-6 fatty acids on lymphoma progression in TLL mice.

Results and discussion

The basis for the experiments in this thesis is the TLL mouse model. This mouse strain developed unintentionally in our laboratory back in 1995 during the work on a project of immunoglobulin heavy chain ($V_{H)}$ gene replacement. Embryonic stem cells (ES cells) from the mouse strain 129/SV were transfected with a targeting vector containing the exon and upstream flanking region of one immunoglobulin heavy chain gene (V_HX24) joined together with the downstream flanking region of another immunoglobulin heavy chain gene (81X V_H) and the Neo resistance and thymidine kinase genes. Targeted ES cells were identified by Southern blot and injected into blastocysts of C57BL/6 (B6) mice. Blastocysts were transplanted into the uterus of pseudopregnant females and offspring derived from ES cell origin were selected on the basis of coat color (black for C57BL/6 and agouti for 129/SV).

Eight true chimeras were obtained and during a period of 3-8 months all eight chimeras died unexpectedly showing a heavy tumor burden in most lymphoid organs. As described below, it was shown that the targeting vector itself had nothing to do with the tumor phenotype and eventually we showed that the ES cells used for the original targeting experiment carried multiple integrations of Moloney murine leukemia virus. The tumor bearing mice were established as a unique mouse strain and further characterized. Initially, as the cause of the tumor phenotype was unknown, a large breeding was carried out so as not to loose the observed phenotype. Later in the project, when MMLV was found to be the cause of the phenotype, the mice could be screened for the MMLV integration and the breeding could be narrowed.

Early on in the project we could show that tumor cells were readily transferrable to immunocompetent hosts, and in the transfer setting recipient mice rapidly and reproducibly developed uniform tumors.

Paper I

Before the tumors developed in the generated chimeras, four of the males were bred to B6 females. Twelve litters with a total of 81 mice were obtained and 9 were excluded as being B6 homozygous by their coat color. The remaining 72 mice were genotypically F1 (129SVxB6). 71% (51 out of 72 mice) of the F1 progeny developed tumors before the age of 30 weeks. In the

next backcross generation (F2), 170 mice were monitored for 50 weeks and 46% developed tumors.

Tumor characteristics

Upon gross examination, the mice showed enlargement of lymph nodes, spleen, liver, thymus and kidneys. Histopathologic analysis showed tumors to be of lymphoid origin with the morphological appearance of medium-sized monotonous lymphoblasts (Figure 6). Numerous mitotic figures were seen and the tumors showed a very high proliferation with S-phases ranging between 25-35%. Tumor infiltrates were also seen in bone marrow and lungs. Immunophenotypic characterization of the lymphoma revealed it to be of T-cell origin with expression of CD4 and/or CD8 and T-cell receptor (TCR) αβ. No expression of B cell markers was found. As tumor cells were present both in bone marrow and as bulky lymphomas in the periphery, the mouse model was designated T-cell lymphoma/leukemia (TLL). The lymphomas of TLL mice were monoclonal as analyzed by Southern blot analyses with a probe specific for the Cβ gene of the TCR.

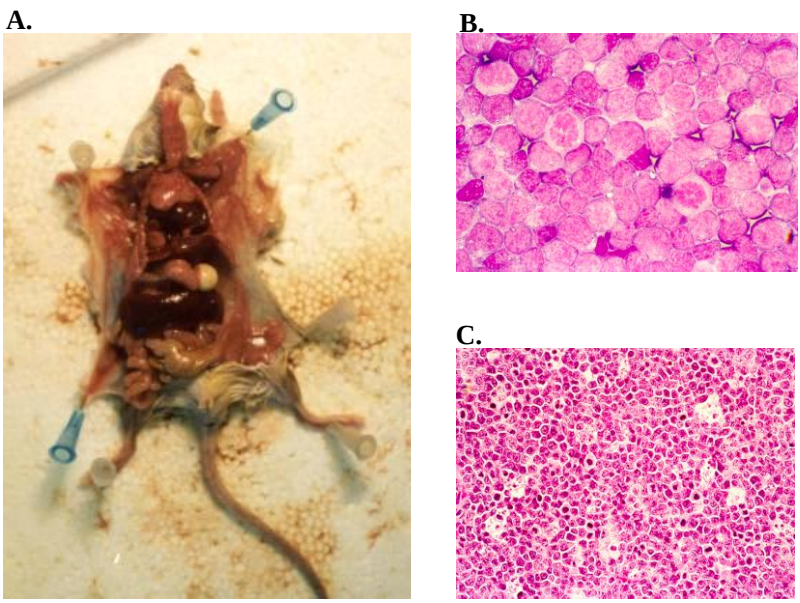

Figure 6. A. Gross examination of a TLL mouse with developed lymphoma showing enlargement of spleen, liver, lymph nodes and kidneys. (Photo B. Eriksson). **B**. Bone marrow showing infiltration of lymphoblasts **C**. Section of spleen in lower magnification showing the typical starry sky pattern of heavy proliferating tumors.

A vast majority of lymphomas had the immunophenotype of mature peripheral T cells when analyzed with flow cytometry using a large number of cell surface marker antibodies. This is in contrast to many other T-cell lymphoma models which display more immature immunophenotype [160-163].

When trying to define the human counterpart to the TLL model, we found it to resemble the HTLV-1 driven A-TLL, acute form. However, the A-TLL cells frequently express CD25, which is negative on TLL cells. Human T-ALL/LBL can be stratified into different stages of thymocyte development and the medullary type has an immunophenotype that resembles that of TLL cells. Both A-TLL and T-ALL/LBL has the aggressive clinical behavior that characterizes the TLL mouse model. Which one of the two human diseases that is the true counterpart of our model is not fully elucidated.

TLL-the explanation?

When further backcrossing mice with the TLL phenotype to B6 mice we showed that the lymphoma phenotype segregated from the targeted immunoglobulin heavy chain locus. Mice carrying the targeted locus as well as mice lacking the mutation developed lymphomas with the same incidence.

While further characterizing the TLL lymphomas, we attempted to find an explanation for the observed phenotype. Karyotyping of tumor cells showed no major translocations or deletions of chromosomal DNA. At about the same time as the TLL mice were developed, the spontaneous generation of T-cell lymphomas was reported in mice heterozygous for a transcription factor called Ikaros. The Ikaros +/- mice were reported to have a similar phenotype as the TLL mice [160]. We examined whether the same mutation had occurred in our mouse strain. However, Southern blot analysis using part of the Ikaros gene as a probe showed no alterations of the Ikaros gene in tumor cells or tail DNA from TLL mice. Further attempts to identify the genetic elements causing the TLL phenotype is described in paper II.

Paper II

In paper II we describe the further characterization of the TLL mouse and the finding of Moloney murine leukemia virus (MMLV) proviral insertions as a plausible explanation for the lymphoma development.

MMLV-the explanation!

We used a differential screening approach modified from Brady et. al. in order to identify genes upregulated in lymphoma cells compared to normal T lymphocytes [164]. A cDNA library from CD4+ T lymphocytes from normal thymus tissue and one from CD4+ lymphoma cells from a TLL tumor were generated and were compared in the differential screen. Clones representing differentially expressed cDNAs were selected and grouped into 48 groups based on restriction fragment length polymorphism (RFLP). Representative clones from each group were sequenced and 42% of the clones were found to be derived from MMLV. Using the MMLV U3LTR probe kindly provided by Professor A. Berns, Netherlands, we performed a Southern blot analysis revealing multiple integrations of MMLV in the ES cell clone used for the initial V_H gene targeting experiment. How and when the infection of the ES cells occurred is not known but related ES cell clones derived from the same source clone were negative for MMLV integrations.

Multiple MMLV integrations were seen in tail DNA from early backcrossed offspring but over time individual integrations were lost during backcrossing to B6 female mice. Two substrains of TLL mice with different but distinct patterns of MMLV integrations on Southern blot were kept after more than 10 generations of breeding TLL mice to normal B6 mice.

Retroviral etiology to hematologic malignancies is common in laboratory mice as described previously and the viruses have multiple roles in lymphoma/leukemiagenesis [54]. We next wanted to elucidate the mechanism by which MMLV cause lymphomas in TLL mice.

Identification of MMLV insertion sites

Retroviral tagging experiments have been conducted to identify new genes or genetic loci contributing to tumor initiation and progression [60-62, 165], facilitated by the completion of the mouse genome sequence in 2002 [166]. Different PCR-based techniques have been used and have allowed researchers to conduct large scale experiments. A large number of common proviral insertion sites have been indentified and have lead to the identification of novel genes and pathways in the oncogenic process.

We used the Splinkerette-PCR technique [167] to identify genomic sequences flanking the MMLV insertion sites in germline of TLL mice. The technique enables PCR amplification of DNA sequences that lie between a single known primer and a nearby restriction site. The amplified fragment has MMLV sequence at one end and unknown genomic flanking sequence at

the other end. We compared the obtained sequences with the mouse genome sequence database from Celera® (a company that in april 2001 completed the first assembly of the mouse genome) to position the integrations and possibly identify genes affected by the proviral insertion and contributing to lymphomagenesis. In the two TLL substrains, the chromosomal localization of the MMLV integration was found to be on chromosomes 2 and 14 and the substrains were denoted TLL-2 and TLL-14 (Figure 7). Sequencing analysis showed no deletions of chromosomal DNA at the proviral insertion sites in the mouse genome, and the proviral genome showed 97% homology to the MMLV sequence reported in GenBank (the National Center of Biotechnology Information genetic sequence database, an annotated collection of all publicly available DNA sequences).

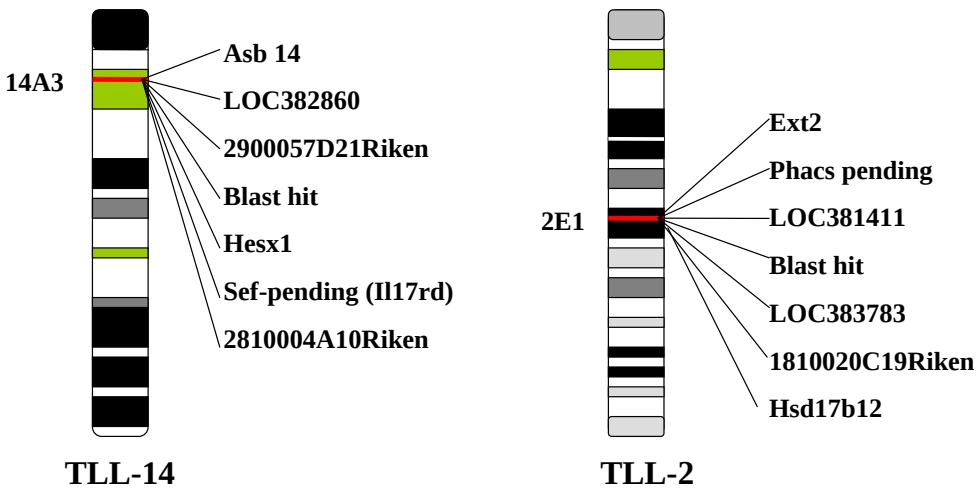

Figure 7. MMLV integration sites on chromosome 2 and 14 and flanking genes or putative genes. BLAST hit= the location of the MMLV integration.

Six genes or putative genes located within approximately 100 kilobases up- or downstream the MMLV insertion site on chromosome 14 were examined for altered expression using Northern blot, RT-PCR and TaqMan real-time PCR techniques. The genes were chosen based on their proximity to the MMLV integration site and for *Sef (Il17rd)* also based on the previous known characteristics of the gene product. *Sef* (*Il17rd)* is an inhibitor of receptor tyrosine kinase signaling where downregulation is seen in a variety of human cancers [168-169]. No alterations in gene expression were seen between wild type B6 mice and TLL-2 or TLL-14 offspring for the genes examined. Since

we failed to demonstrate any change in gene expression for the candidate genes on chromosome 14, we decided not to proceed with the same approach for the genes flanking the MMLV integration on chromosome 2.

One important step in MMLV lymphomagenesis is the generation of MCF viruses as described above, allowing additional integrations of proviral DNA in the target cells. We performed a Southern blot of 10 lymphoma derived DNA samples from each substrain and compared the hybridization pattern with corresponding tail DNA from the mice. This revealed several additional MMLV integrations in the lymphoma derived DNA, demonstrating that additional rounds of infection had occurred with additional proviral integrations at different sites in the host cell DNA. The number of additional integrations did not differ between TLL-2 and TLL-14 substrains. We demonstrated in average 10 additional integrations in each tumor, ranging from 5-20. We also demonstrated the presence of viral RNA sequences in serum samples from TLL mice showing that the mice were viremic during a period preceding lymphoma development.

We then concluded that the localization of the proviral integrations carried in germline of TLL mice is probably not decisive for lymphomagenesis as long as the integration lies in an actively transcribed region. The presence of MMLV in germline is instead thought to be a source of infectious competent retrovirus enabeling infection and integration in the genome of T lymphocytes during their maturation and by the multistep process described previously leading to lymphoma development.

TLL-2 and TLL-14 substrains

Although we conclude that the position of proviral integration within the genome is not crucial for lymphomagenesis, we observed some differences between the two TLL substrains. We thus decided to compare the two TLL substrains TLL-2 and TLL-14 with regard to lymphoma frequency and immunophenotype of the lymphoma. In this study we also included mice carrying both integrations in germline, denoted TLL-2/14.

The spontaneous lymphoma frequency in TLL-14 mice was found to be significantly higher than in TLL-2 mice; 60% compared to 37% after 12 months observation time. The TLL-2/14 mice showed 74% lymphoma frequency after the same time period. The mean age of onset of disease was significantly lower for TLL-14 mice, 7 months compared to 9 months for TLL-2. TLL-2/14 mice showed an even earlier time of onset, around 5 months. When examining mice homozygous for the integration on chromosome 14, the lymphoma frequency was significantly higher than in

heterozygous mice suggesting that the number of proviral copies in germline is affecting disease outcome.

The lymphomas in TLL-14 and TLL-2 mice were macroscopically similar with predominant involvement of spleen, liver and lymph nodes. All lymphomas were immunophenotyped by FACS analysis using antibodies for CD4 and CD8. For the TLL-14 strain and the combined strain, the majority of lymphomas expressed either CD4 or CD8 on the surface, characteristics of a mature T-cell phenotype. In contrast, lymphomas arising in the TLL-2 substrain showed an immature immunophenotype with CD4/CD8 double positivity or double negativity.

The differences in lymphoma frequency and maturation status of the lymphomas observed between the TLL-2 and TLL-14 substrains have several plausible explanations. The observed difference could be due to the transcriptional activity in the chromosomal regions on chromosomes 2 and 14. Higher activity may lead to higher proviral expression and the T-lymphotropic virus can infect T cells during crucial periods of development and initiate the multistep process of lymphomagenesis. Higher transcriptional activity can in turn be a result of more accessible chromatin structure and/or DNA methylation. The timepoint of MMLV viremia during embryonic or postnatal development of the mice can possibly affect the virus ability to infect target T-lymphocytes at different stages of development, leading to the observed differences in maturation status of the lymphomas.

Paper III

TLL transfer model

In this paper, we used the TLL transfer model to test the effect of a selective COX-2 inhibitor, celecoxib, on lymphoma progression. The transfer model was developed for the purpose of testing different therapies and has been previously used with an angiogenesis-inhibitor, TNP-470 [170].

When TLL tumor cells are injected intravenously into syngenic recipients (C57BL/6 mice (B6)), the tumor phenotype develops after 9-10 days with the recipient's lymphoma showing identical immunophenotype as the donor disease. The system has the advantage over spontaneous tumors in its predictability and shorter time to disease onset. To ensure that all recipients in the experiment received identical tumor cells, aliquots of TLL cells from a mouse spleen affected by lymphoma were frozen, then thawed and injected intravenously into B6 mice (these are referred to as donor mice). When

tumors developed in donor mice, sufficient numbers of lymphoma cells to inject multiple recipients were obtained and the cells were used directly without freezing. In this model, we estimated tumor burden based on spleen weight and percentage of CD4 positive T cells in the spleen. Macroscopically, the appearance of the lymphoma was uniform with heavily enlarged spleen, liver and lymph nodes at time of sacrifice. Histopathological examination of each tumor was not done as we had earlier concluded the uniformity of the disease.

Celecoxib treatment

Based on previous reports of celecoxib derivatives showing promising activity against B-cell lymphoma both *in vitro* and *in vivo* [154], we wanted to examine the effect of celecoxib in T-cell lymphoma, an area where data have been lacking. We took advantage of the TLL transfer model to investigate the potential of celecoxib alone and in combination with the well established antitumor agent cyclophosphamide as a putative novel agent for T-cell lymphoma therapy.

Despite the fact that many of celecoxib´s antitumor activities are exerted independently of the COX-2 pathway, we found it relevant to first investigate the functionality of the COX-2 pathway in our model system [147, 156]. We analyzed prostanoid levels in spleens and lymph nodes from mice with T-cell leukemia/lymphoma compared to the same tissue from healthy controls. PGE_2, PGD_2, PGF_{2a}, TXB_2 and prostacycline levels were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and, interestingly, were found to be lower in spleens from TLL mice than in spleens from healthy mice. The prostanoid levels in lymph nodes were not substantially different between healthy and lymphoma affected mice.

From these results, it was concluded that the COX-2 pathway is not necessary for TLL-lymphoma growth and thus not relevant as drug target. Since multiple pathways other than COX-2 have been associated with the antitumor effects of celecoxib, we still found it relevant to conduct the therapeutic studies. Mice injected with TLL cells were treated with celecoxib by oral gavage in 10 mg/kg, 30 mg/kg and 60 mg/kg doses daily from day 1 after cell transfer until the mice showed signs of disease and were sacrificed. The mice were sacrificed.at the same time point as untreated controls at day 10. Independent of celecoxib dosage, all mice showed the same tumor burden as measured by spleen weight and CD4 positive T cells in the spleen.

A concern was that the TLL transfer model is a rapidly developing tumor model with aggressive clinical behavior and celecoxib alone might not be

sufficient to affect the disease. We therefore combined celecoxib with cyclophosphamide, a conventional antitumor agent commonly used for lymphoma treatment, to evaluate if additional effects could be seen compared to monotherapy with cyclophosphamide. Combination of celecoxib with chemotherapy or radiotherapy has also been reported with interesting results in other preclinical tumor models [171-173].

In initial experiments, we established that treatment with cyclophosphamide 100 mg/kg intraperitoneally at day 4 after TLL cell transfer postponed the progression of disease by about 10 days. This regimen was chosen and combined treatment was given with celecoxib orally 60 mg/kg daily from day 1 after TLL cell transfer until the mice were sacrificed. In the study, four groups of mice (n=6/group) were included (all groups receiving the same amount of TLL cell transfer iv); one group receiving only celecoxib, one group receiving only cyclophosphamide, one group receiving combination treatment and a control group without treatment. The group of mice receiving only celecoxib and the untreated group were sacrificed at day 11 after transfer. The cyclophosphamide monotherapy group and the combination treated group were both sacrificed at day 22. At the time of death all mice showed clinical signs of disease and macroscopic examination revealed heavy lymphoma burden. No difference was seen between mice treated with cyclophosphamide alone compared to combination treated mice with respect to spleen weight or percentage of CD4 positive spleen cells. Since we could not demonstrate any *in vivo* effects of celecoxib in our studies we did not go further with functional studies of presumed cellular pathways affected by celecoxib.

One limitation of this study is the lack of pharmacokinetic investigations, so the lack of effect cannot be related to plasma concentrations of celecoxib. However, other investigators have shown that doses ranging between 10-60 mg/kg in mice are sufficient to inhibit tumor growth and 100 mg/kg gave substantial toxicity (Table 3).

Efficacy of Celecoxib in animal models of cancer

Study	Tumour type	Celecoxib dose	DMo
Kardosh [154] [154]	Burkitt lymphoma	150 mg/kg/d, p.o.	TGI
Grösch [174]	colon	10-100 mg/kg/d, p.o.	TGI
Blanquicett [171]	pancreatic	20 mg/kg/d, p.o.	TGI;additive with RT/CT
Shaik [172]	NSCLC	150 mg/kg/d, p.o.	TGI;additive with CT
Di Cello [175]	uterine	120 mg/kg/d, p.o.	TGI
Tong [176]	retinoblastoma	250 mg/kg/d, p.o.	no
Farooqui [177]	mammary	30-100 mg/kg/d, p.o.	Survival ¤Antimetastatic
Johnsen [178]	neuroblastoma	10 mg/kg/d, p.o	TGI
Cui [179]	hepatocellular	12.5-50 mg/kg/d, p.o.	Tumour frequency;TGI
Klenke [180]	NSCLC	30 mg/kg/d, s.c.	TGI;Antiangiogenesis
Zheng [181]	prostate	10 mg/kg/d, i.p.	TGI;additive with statin
Dovedi [182]	urothelial	20-100 mg/kg/d, s.c.	TGI;additive with BCG
Johansson (present study)	T-cell lymphoma	10-60 mg/kg/d, p.o.	no

Table 3. Efficacy of celecoxib in animal models of cancer and dosage used. NSCL (non-small cell lung cancer), CT (chemotherapy), RT (radiotherapy), DMo (disease modifying effect), TGI (tumor growth inhibition). ¤= 50% died of toxicity after 100 mg/kg.

Available animal data (Table 3) also suggest that tumor origin could be important for the effect of celecoxib with epithelial tumors showing growth inhibition at clinically relevant doses of celecoxib while non-epithelial tumors might not respond in a similar way.

We conclude that in our model system celecoxib seems to lack effect against the highly aggressive T-cell leukemia/lymphoma. In this study, we did not examine if other known targets for celecoxib's antitumor effects, beside COX-2 inhibition, were affected by the treatment.

Paper IV

The growing scientific and public interest in dietary factors and their effect on cancer together with the apparent lack of consensus in this matter inspired us to investigate the role of dietary omega-3 and omega-6 fatty acids in our spontaneous TLL mouse model. The model is described in Paper I and II.

Omega-3/omega-6 and lymphoma development

Male TLL-14 mice were bred to B6 females and the resulting litters, all heterozygous for the MMLV insertion, were assigned to one of three dietary groups when weaned at three weeks of age. 63 mice were fed a diet supplemented with 9% menhaden fish oil and 1% corn oil (omega-3 rich diet), 67 mice were fed a diet supplemented with 10 % corn oil (omega-6 rich diet) and 20 mice received standard laboratory mouse chow with no additives. The diets were continued until the mice were sacrificed due to symptoms of lymphoma (rugged fur, abdominal swelling and decline in general condition) or until 12 months of age when the study was terminated. The 12 month termination point was chosen because the spontaneous lymphoma frequency increases in the aging C57BL/6 mice and after 12 months of age the specific contribution of the MMLV insertion on lymphoma frequency might be hard to evaluate [183]. All mice were subjected to autopsy and mice macroscopically having heavily enlarged spleen, liver and lymph nodes were scored dead by lymphoma. A few mice died from other causes during the study period. No histopathologic examination of affected organs to confirm presence of lymphoma were made, since all our previous experience with the TLL model indicate high consistency between macroscopical and histopathological findings. All macroscopic scoring of tumors were made by the same investigator (Åsa Larefalk).

We could show that dietary supplementation with omega-3 fatty acid significantly delayed disease onset between 5-8 months of age compared to standard mouse chow with p=0,022 (5 months), p=0,04 (6 months), p=0,037 (7 months) and p=0,043 (8 months). However, at 12 months (termination of study) no difference between diet groups was seen (Figure 8). There was no significant difference in disease onset between corn oil supplemented group and fish oil supplemented group or between corn oil supplemented and standard chow groups. At macroscopic gross examination no differences between mice from the different dietary groups in extent of lymphoma could be seen at autopsy.

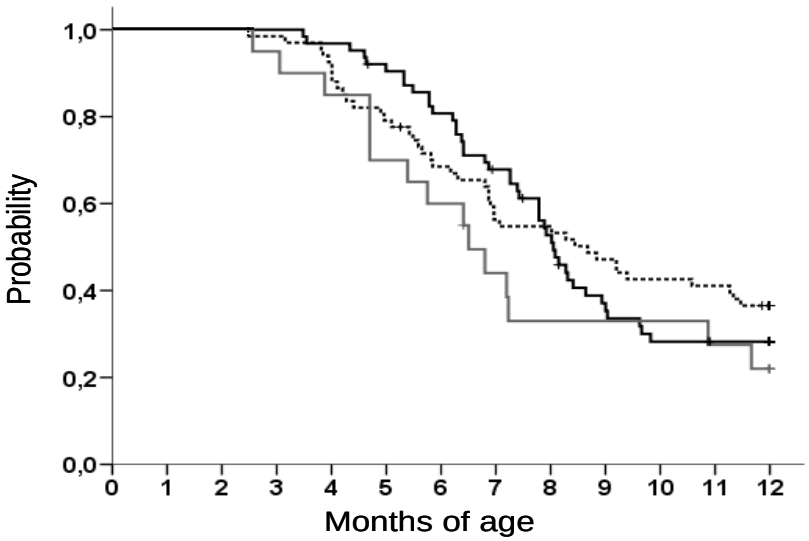

Figure 8. Effects of dietary fatty acid supplementation on probability of lymphoma-free survival in TLL mice. Solid line=fish oil supplemented diet (n=63), dotted line=corn oil supplemented diet (n=67), grey line= standard mouse chow (n=20).

Omega-3/omega-6 and immune cell populations

When the first observations of delay in disease onset in mice fed an omega-3 rich diet were made, this raised questions of underlying mechanisms. Since composition of dietary fat intake is known to affect cells of the immune system in both mice and men [79-81], this led us to initiate a parallel study with the aim to investigate the influence of omega-3 and omega-6 dietary supplement on the development of lymphoid cell populations in healthy mice.

Thirty C57BL/6 mice per group were fed the respective diets; menhaden fish oil supplemented (omega-3 rich), corn oil supplemented (omega-6 rich) or standard mouse chow from the day of weaning at three weeks of age until sacrificed. Ten mice per group were sacrificed at 2, 4 and 6 months respectively and spleen, thymus and bone marrow were collected and analyzed with flow cytometry for alterations in the composition of the major lymphoid subsets; T and B lymphocytes at different maturation stages and NK cells.

In cells from bone marrow the proportion of CD43 and B220 positive cells was determined, CD43 being a marker for immature B cells and B220 a marker for all B-lineage cells. The fish oil supplemented experimental group

showed a significantly higher proportion of B220 positive cells compared to both the corn oil supplemented and standard diet groups. This was seen in all examined mice aged 2, 4 and 6 months but was less pronounced at increasing age.

Thymocytes were analyzed for expression of CD4, CD8 and also for markers of early T-cell development (CD4/CD8/CD25/CD44). The CD4/CD8/CD25/CD44 pattern characterizes the four different stages during the double negative developmental stage. No significant differences in the thymic composition of doublenegative thymocytes of the four stages could be seen. Neither could we demonstrate any significant differences between CD4/CD8 single or doublepositive populations between the three dietary groups.

In splenocytes, we analyzed the T- and the B-cell populations as well as presence of NK cells. TCRγδ T cells have been demonstrated to have immunoregulatory properties and could be of particular interest [13]. No significant differences could be demonstrated between the dietary groups with regard to the splenic T-cell populations examined. For NK cells (based on NK1.1 marker expression on the cell surface), significantly lower percentages could be seen in fish oil fed group compared to corn oil fed group at all ages. A difference in NK cell percentage between control group and fish oil group could be seen at 2 months of age and between the control group and the corn oil group at 6 months of age.

We could also demonstrate differences in the splenic compartments of the B lineage. Marginal zone B cells, defined by positive staining with IgM and CD21 but negative staining for CD23, were significantly decreased in the fish oil fed group compared to the corn oil and standard chow groups at all ages. For follicular B cells (IgM+CD21+CD23+) there was a tendency towards increase of this cell population in the fish oil fed group compared to corn oil fed group at 4 and 6 months but it did not reach significance.

In conclusion, in our experimental setting some changes in the composition of immune cell compartments could be detected in healthy laboratory mice with respect to dietary intake of omega-3 and omega-6 fatty acids. Determining the exact mechanisms for this and whether other sub-specialized populations of immune cells were affected by diet would require further and more detailed analyses.

Previous studies have examined the effect of fatty acid supplementation on leukocyte numbers and subsets. A study from Thies and coworkers found no differences in the total number of leukocytes, lymphocytes, T lymphocytes or

NK cells in peripheral blood from humans after adding different PUFAs to the diet for 12 weeks [184]. We have found no previous studies characterizing the effect of dietary omega-3 or omega-6 PUFAs on the balance between different B-cell populations *in vivo*. However, many studies have compared the effect of different diets on lymphocyte function and NK-cell activity [74, 185-187]. Lowering the total amount of fat in the diet will enhance lymphocyte responses. Other studies have suggested that fish oil supplementation decreases the cell mediated immune responses by decreased activity of antigen presenting cells and by decreased production of certain cytokines and their receptors [186, 188].

It is known that modifying the fatty acid composition of the diet leads to increased levels of the supplemented fatty acid in the immune cell membranes in a dose dependent manner [73, 189]. Increased access to omega-3 PUFAs typically increases the amount of omega-3 PUFAs in the cell membrane of immune cells at the expense of arachidonic acid. This in turn leads to a shift in production of eicosanoids from an omega-6 pro-inflammatory profile to an omega-3 anti-inflammatory profile.

In humans, low NK-cell activity is associated with increased cancer risk [190]. NK cells both act as effector lymphocytes of the innate immune system and as negative regulators of inflammation and immune response. Suppression of NK cells could therefore lead to both increased and decreased cancer risk. Increased cancer risk due to lack of direct cytotoxic effect on transformed cells and decreased risk if impaired number or function of NK cells with immunoregulatory functions leads to increased activity of other immune cell subsets with influence on tumor progression. The latter mechanism might be an explanation to our observations that supplementation with omega-3 fatty acids leads to lower NK-cell numbers as well as delay in lymphoma development.

The skewing of B-cell populations seen in both bone marrow and spleen in fish oil supplemented mice could delay lymphoma development by unknown immunological mechanisms. The transient delay of lymphoma growth seen after supplementation by omega-3 PUFAs could, however, also be the result of direct anti-proliferative effects by the omega-3 PUFAs on the malignant cells, or of other unknown paracrine effects.

In our study, the fish and corn oil supplemented diets were higher in the amount of total fat and calories than the standard diet. As mentioned previously, total fat intake can affect both cancer incidence and immune functions. However, in a recent case-control study no significant association was seen between energy intake, fat intake and lymphoma development

[107]. A similar lack of association between total fat intake and lymphoma was reported for mice prone to develop lymphoma [191]. Total fat was also the same for the fish oil and the corn oil diets.

In our study, we could not demonstrate an effect of omega-6 supplementation of lymphoma progression. Some previous studies have indicated tumor growth-promoting effects of omega-6 PUFAs, but the effects on lymphoid malignancies are unclear.[192].

Conclusions

- The T-cell malignancy seen in the TLL mouse is an aggressive T-cell leukemia/lymphoma with similarities to human lymphoblastic lymphomas with regard to morphology, immunophenotype and cell proliferation rate.

- The presence of Moloney murine leukemia virus in germline of the TLL mouse is a requirement to develop the leukemia/lymphoma phenotype, but the mechanisms are not fully elucidated.

- The two substrains of TLL mice (TLL-2 and TLL-14) differ significantly with respect to frequency of the disease and also display differences in immunophenotype of the tumors suggesting that position of the proviral integration can affect different properties of the disease.

- The selective COX-2 inhibitor celecoxib lacks significant effect on lymphoma progression in mice transplanted with TLL cells, both as monotherapy and in combination with cyclophosphamide.

- A diet rich in omega-3 PUFAs significantly postpones the onset of T-lymphoblastic disease in the TLL mouse but the effect is transient and does not affect long-term survival.

- A diet rich in omega-3 PUFAs leads to alterations in proportion of selected lymphoid cell populations; an increase of mature B cells in bone marrow, a decrease of NK cells in the spleen and an increase of marginal zone B cells in the splenic white pulp in C57BL/6 mice.

Future perspectives/concluding remarks

Lymphomas are tumors that generally respond well to traditional therapies such as chemotherapy and radiotherapy. However, for the patients that do not respond, or when tumors recur, new therapeutic approaches are warranted. In recent years, many new anti-cancer compounds have reached the market or are on their way towards approval. For aggressive T-cell lymphomas that are relatively rare and thus harder to study in large scale clinical trials, pre-clinical studies are important to screen the new compounds for potential effect against the disease.

The TLL mouse model has proven to be a useful tool in studies of lymphomagenesis and lymphoma treatment. Other retrovirally induced lymphoma models exist in mice, but often with the requirement to inoculate newborn mice with infectious virus. Additional murine tumor models build on introducing tumor cells into immunocompromised hosts. The defective immune response in the host can affect the parameters studied, making results difficult to interpret. TLL mice are immunocompetent and require no manipulation for the lymphoma to develop. The studies reported in this thesis are just a sample of different applications for the model. The spontaneous model is suitable for preventive studies and the transfer models works well for testing new therapies or combinations of therapies.

The complex mechanisms of eicosanid production and function in immune responses and tumorigenesis are a challenge to researchers. The effect of diet on these pathways also make it an area of great public interest, and studies show that complementary and alternative medicine use (including dietary supplements) is common among cancer patients [193]. It is of great importance that the scientific community provide the public with reliable data, so that recommendations concerning diet and other preventive strategies for cancer are evidence based. Conflicting results between animal and human studies in dietary experiments are, however, common. This is likely due to the difficulty in translating a controlled experimental setting in a laboratory to real life with all its dietary temptations, most of them with “wrong” omega-3/omega-6 balance! It is still important, however, to elucidate new concepts in the laboratory but confirming them in real life clinical settings before making general statements on how our life-style affects disease development.

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