From the Department of Medicine
Karolinska Institutet

GENETIC MODIFICATION OF HUMAN NATURAL KILLER CELLS AND POSSIBLE APPLICATIONS THEREOF

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Stockholm 2006

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Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN: 91-7140-867-3

Carpe Diem..

To my family

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1. SUMMARY

Natural killer (NK) cells are dependent on the presence of interleukin-2 (IL-2) for their survival and for their cytotoxicity against tumor cells. Currently, for *in vitro* expansion of NK cells from peripheral blood mononuclear cells (PBMCs), IL-2 is of great importance. Moreover, during the infusion of NK cells back into patients, systemic IL-2 administration enhances their proliferation and immunological properties. However generic IL-2 administration is frequently impeded by undesirable side effects, such as high toxicity and undesired activation of neighboring cell populations. The latter is extremely unfortunate especially after allogeneic hematopoietic stem cell transplantation, where activation of, e.g., T cells and Treg cells could lead to GVHD and immunosuppresion. The aim of this thesis was to investigate whether NK cell activity can be improved by transduction with IL-2 with regard to *in vitro* expansion leading to the independence from exogenous IL-2 administration and thus a more promising cancer therapy solution. However, since secreted interleukins have proven to be non-specific, leading to undesirable toxic side effects, we reasoned it would be better if interleukins were expressed in a localized and controlled manner. Initially, as proof of principle, the IL-2-dependent NK-92 cell line was transduced with wild type IL-2 (IL-2WT) or an IL-2 vector targeted to subcellular organelles, including ER (IL-2ER) (Paper I). NK-92 cells were also transduced with GFP as a positive control. When populations of modified and non-IL-2 modified NK-92 cells were mixed, it was shown that the ER-targeted IL-2 did not support the growth of neighboring NK-92 non-IL-2-modified cells. This indicated that, in the case of endoplasmic reticulum targeting, the IL-2 does not leak to the supernatant. Next, in order to establish an efficient protocol for retroviral transduction of primary NK cells, PBMCs, including NK cells were transduced with GFP on several timepoints during a 21-day expansion, with day 5 showing the best transduction rate (Paper II). In order to test the above IL-2 constructs *in vivo*, an animal model had to be established, where cells could be monitored (Paper III). For this purpose 5T33MM cells were transduced with GFP, injected into C57Bl/KaLwRij mice, and monitored for homing and mice survival purposes. Finally this GFP MM mouse model was used to assess the therapeutic potential of IL-2-induced NK cells (Paper IV). NK cells separated from single-cell suspensions derived from mouse organs, and activated with IL-2, were shown to eliminate syngeneic myeloma cells *in vitro* and to prolong the survival of 5T33MMGFP^{+}-immunized mice *in vivo*.

2. LIST OF PUBLICATIONS

The following publications are referred to in this thesis by their Roman numerals.

I. Konstantinidis K.V, Alici E, Aints A, Christensson B, Ljunggren H.G and Dilber M.S. (2005) Targeting IL-2 to the endoplasmic reticulum confines autocrine growth stimulation to NK-92 cells. *Experimental Hematology*, **33**(2): 159-164.

II. Guven H.*, **Konstantinidis K.V.***, Alici E, Aints A, Abedi-Valugerdi M, Christensson B, Ljunggren H.G and Dilber M.S (2005). "Efficient and easy-to-use gene transfer into primary human natural killer (NK) cells by retroviral transduction". *Experimental Hematology*, **33**(11): 1320-1328.
* contributed equally.

III. Alici E, **Konstantinidis K.V,** Aints A, Dilber M.S and Abedi-Valugerdi M (2004) Visualization of 5T33 myeloma cells in the C57BL/KaLwRij Mouse: establishment of a new syngeneic murine model of multiple myeloma. *Experimental Hematology,* **32**(11): 1064-72.

IV. Alici E., **Konstantinidis K.V.**, Aints A, Gahrton G, Ljunggren H.G and Dilber M.S. Anti-myeloma activity of adoptively transferred IL-2 activated natural killer cells in the C57Bl/KaLwRij mouse model Manuscript

3. LIST OF ABBREVIATIONS

ASCT, autologous stem cell transplantation
BM, bone marrow
CINK, cytokine induced natural killer cells
DLI, donor lymphocyte infusions
ER, endoplasmic reticulum
FACS, fluorescence activated cell sorting
GFP, green fluorescent protein
GvHD, graft versus host disease
HLA, human leukocyte antigen
HSCT, hematopoietic stem cell transplantation
HS, human serum
IFN-γ, interferon gamma
IL-, interleukin
IL-2WT, interleukin 2 wild type (secreted form)
IL-2ER, interleukin 2 endoplasmic reticulum retained expression
i.v., intravenous
IU, international unit
IRES, internal ribosome entry site
HSV-tk, herpes simplex virus thymidine kinase
LAK cells, lymphokine activated killer cells
MHC, major histocompatibility complex
MM, multiple myeloma
NK cells, natural killer cells
NKT cells, natural killer-like cells
OKT-3, othoclone monoclonal antibody against CD3
PBMCs, peripheral blood monocuclear cells
PBS, phosphate buffered saline
s.c., subcutaneous
SCID, severe combined immunodeficient
TNF, tumor necrosis factor

4. GENERAL INTRODUCTION

4.1 The immune system

The study of the immune system dates all the way back to ancient Greece, where the historian Thucydides describes the importance of immunity during the plague epidemy of 430 B.C in Athens[1]. More specifically he mentioned that 'the same man was never attacked twice, at least never fatally'. However, it took several centuries until the science of immunology was established in 1796 with the discovery of the smallpox vaccine by Edward Jenner. Since then, there have been many developments in this field. Notably, during the last century, no less than 23 distinguished scientists have received the Nobel Prize for their lifetime achievements related with 17 discoveries in the field of immunology.

The immune system is the body's complex system of defense against infections and foreign substances. During this section and the following subsections - 4.1.1 and 4.1.2- I refer to the textbook 'Immunobiology – the immune system in health and disease' by Charles Janeway *et al.*,1999 and 'Immunology' by Janis Kuby, 1994[2,3]. The reactions of the immune system to infectious agents are crucial to survival and hence, usually, beneficial for continued health. However, occasionally our immune system can be responsible for the cause of autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis. In those cases, where the immune system attacks its own self, the reaction of the immune system can lead to tissue damage and organ failure.

The immune system can be divided in non-specific (innate-natural) and specific (adaptive-acquired) immunity. For a schematic view see Figure 1.

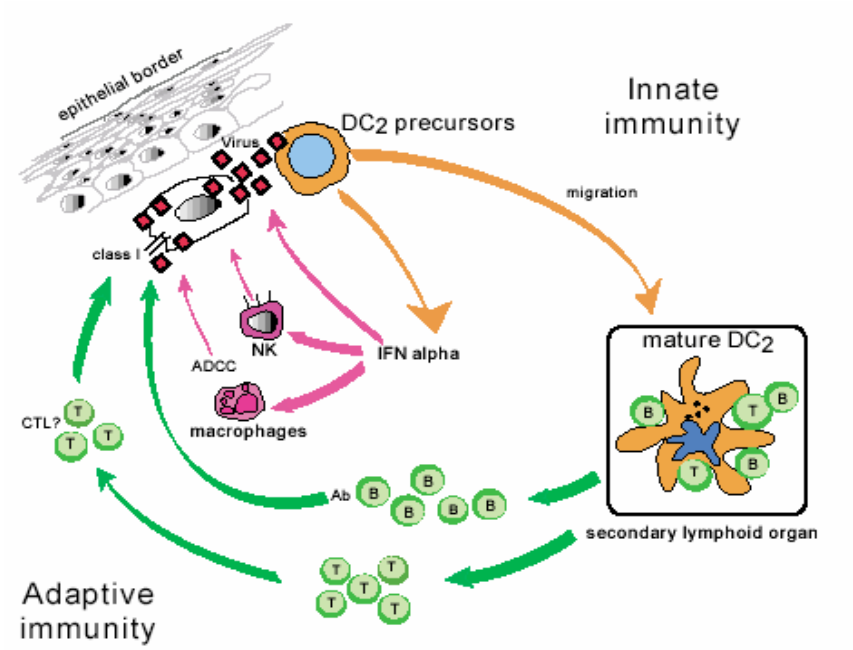

Figure 1. Schematic view of the interactions between innate and adaptive immunity

Nevertheless, as illustrated in Figure 1, the constant interaction of the latter two types of immunity makes their delineation complicated. Both innate and adaptive immunity interact intimately and stimulate each other. Innate immunity may be more primitive, from an evolutionary aspect, but adaptive immunity improves and amplifies the efficiency of natural mechanisms, as well as memorizes and acts quicker next time the same infection is encountered. Finally, innate immune receptors show a high degree of specificity, though different from the adaptive immune receptors T and B cell receptors.

4.1.1 The innate immune system

The innate immune system is the first line of defense against foreign intruders and is responsible for the initial immune reactions before the adaptive system is activated. Its response to infection is non-specific and it does not generate any immunologic memory. The innate immunity consists of cellular and molecular components.

The cellular components of the innate immune system consist of phagocytes conferring resistance to intracellular mediators (macrophages, monocytes, neutrophils), cells releasing inflammatory mediators (basophils, eosinophils, mast cells) and natural killer cells that destroy infected and malignant cells. The properties and functions of NK cells, one of the main protagonists of this thesis, will be described in detail in a section below (Section 4.2).

The molecular components of the innate immune system are the acute-phase proteins that enhance resistance to infection and promote repair of the damaged tissue, the complement system and the cytokines. The immune contribution of the complement is based on a group of serum proteins that are involved in the control of inflammation, the activation of phagocytes and the lytic attack on cell membranes. The complement system that includes more than 30 soluble and cell-bound proteins is divided into three activation pathways; the classical-, the alternative- and the lectin pathway. The two latter ones conform part of the innate immune system, since the complement binds to foreign cell surfaces. In contrast, the classical pathway is activated by specific antibodies, and is therefore part of the adaptive immune response. The cytokines include the interleukins, the interferons, colony stimulating factors and tumor necrosis factors and act as signals of the immune system. The immune cells make more than one cytokine when activated and each individual cytokine can have several functions depending on the cell that it binds to. This multi-functional effect of cytokines is called pleiotropy. However, in order for the cell to respond to a cytokine, it has to express the appropriate receptor.

Innate immunity provides non-specific protection through a series of defense mechanisms. Firstly, the anatomic barriers, the skin and the surface of mucous membranes, prevent the entry of pathogens and thus provide an effective barrier to micro-organisms. The skin, which consists of the epidermis – a thin outer layer- and the dermis – a thick inner layer-, when intact prevents

pathogen penetration and inhibits bacterial growth due to a low pH. Secondly, there are physiological mechanisms; the body temperature inhibits pathogen growth, the interferons induce an antiviral state to nearby binding cells, the lysozyme cleaves the peptidoglycan layer of the bacterial cell wall and the complement system acts as mentioned above. Thirdly, the innate system employs phagocytic mechanisms, where the phagocytes bear several receptors that recognize microbial components and induce phagocytosis, as well as cytokine release. Finally innate barriers are created by inflammatory response. In this latter case, upon tissue damage, bacteria attract macrophages that are triggered to release cytokines and chemokines. In parallel, the inflammatory cells migrate to the tissue, releasing inflammatory mediators that in turn cause pain.

4.1.2 The adaptive immune system

The adaptive immunity has, in contrast to the adaptive immunity, the ability to specifically recognize and eliminate foreign bodies and infected cells. Here, host defenses are mainly mediated by B, T and antigen presenting cells following exposure to antigen, which can exhibit specificity, diversity, immunologic memory, or self/nonself recognition. B and T lymphocytes are derived from a lymphoid stem cell, called the lymphoid progenitor.

The development of T cells takes place in the thymus, a specialized organ, where T cell progenitors mature and differentiate into cells that can kill infected cells or activate other cells of the immune system. The thymus is divided into the following anatomical regions; the subcapsular epithelium, the cortex, the cortico-medullary junction and the medulla. T cell progenitors enter the thymus through endothelial venules and migrate to the subcapsular epithelium, where they start the maturation process by rearrangement of their T cell receptor genes.

During this maturation stage, the developing thymocytes express both the CD4 and CD8 cell receptors. Cells with a receptor that recognize a self MHC class I molecule, receive a survival and a maturation signal that results in a halt of CD4 expression and initiates the gene expression characteristics of cytotoxic CD8 T cells. On the contrary cells that are capable of recognizing a self MHC class II molecule, receive the survival and maturation signal to stop expressing CD8 and to start expressing genes characteristic of helper and inflammatory CD4 T cells. Those thymocytes that are unable to recognize either MHC I or MHC II, or express these molecules too avidly, or recognize self-antigen receive a programmed cell death signal. The surviving T cells migrate from the cortex to the medulla and return at last to the blood stream.

On the other hand B cells develop in the bone marrow (BM) and then migrate to the lymphatic tissue. Each B cell expresses a unique antigen-binding receptor; a membrane bound immunoglobulin on the cell surface. B cells can differentiate into effector plasma cells that secrete large amount of

immunoglobulins and to memory B cells that remain resting in the bone marrow. Plasma cells secrete soluble antibodies that inhibit the binding of microorganisms to the cellular receptor on the target cells. Plasma cells can also activate other components of the immune system, such as the complement, that can result in lysis of bacteria, or enhancement of phagocytosis of complement-coated microorganisms. Memory cells are assigned the task of a faster and more specific immune response during re-infection of the same pathogen.

4.2 Natural killer cells – An overview

Natural killer cells were discovered in the middle 1970s by Rolf Kiessling and colleagues here at Karolinska Institutet in parallel with a research group in the USA[4-7]. While measuring the cytotoxic activity *in vitro*, they observed a murine lymphocyte population with an unusual spontaneous cytotoxicity against certain tumor cells.

NK cells are non-T and non-B cytotoxic lymphocytes that are involved in the killing of tumor cells[8]. NK cells are usually confined to the bone marrow, the spleen and the peripheral blood, but they can also be found in inflammatory tissues due to their response to chemoattractants. In the blood, NK cells are abundant, and comprise 5%-20% of the circulating lymphocytes. When an NK cell contacts its target, it causes the event of cytolysis. The majority of NK cells express the surface antigens CD56, CD16, CD7, CD8 and CD2 and at the same time they express the high and the intermediate affinity IL-2 receptor (IL-2R)[9].

In humans, NK cells develop very rapidly during the ontogeny[10]. They appear during the gestation period, even before the development of T cells. In newborn mice, NK cells lack many of their usual receptors, but only a few weeks of maturation are required till their receptors are fully expressed[11]. NK cells have many different properties [12]. NK cells can e.g. contribute significantly to immunoregulation by secreting high levels of lymphokines, such as the interleukins group. NK cells may also affect the *in vivo* growth control of a tumor and its metastatic speed. The same NK cells that target tumor cells also play a key role in innate immunity to viruses. Furthermore, NK cells contribute to monocyte and granulocyte cell growth, as well as haematopoiesis. In addition, they provide defense against intracellular bacteria[13], control some types of viral infection[14] and contribute to antitumor activity both *in vitro*[6] and *in vivo*[15].

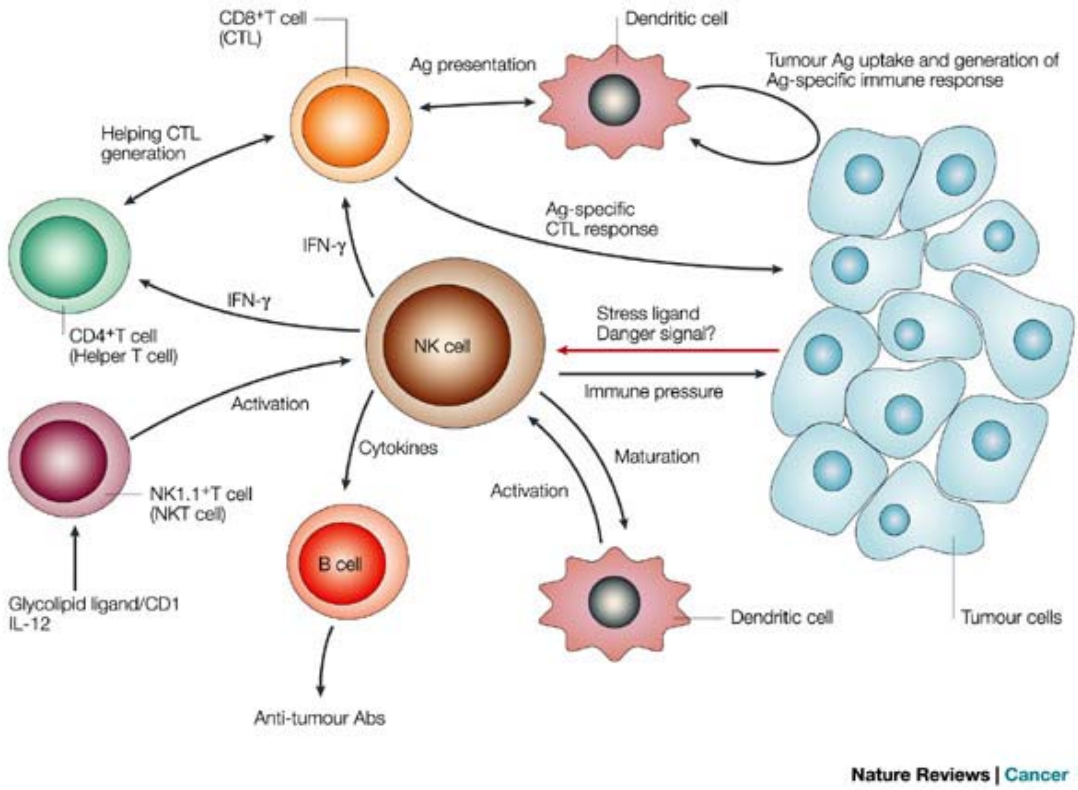

Figure 2. NK cells and the immune responses to tumor cells. Reproduced from Nature Reviews Cancer, Smyth *et al.*, 2002.

4.2.1 Natural killer cell receptors – Activation and inhibition

In order for a human NK cell to be activated, the engagement of a number of receptors is required[16]. During a natural cytotoxic response the following receptors are responsible for NK activation; NKp46, NKp30 and NKp44[17]. Lately a new triggering molecule, NKp80, which is expressed in human NK cells, has been identified[18]. Together these four receptors are part of a group of receptors that are known as natural cytotoxicity receptors (NCRs). NCRs participate in the lysis of many tumor cell lines[19].

The deactivation of NK cells is achieved when inhibitor surface receptors recognize specific MHC class I molecules[16]. The signaling cascade is deactivated through a NK cell inhibitor receptor mechanism. NK cells, according to the 'missing self' hypothesis, identify and eliminate cells that do not express the self major histocompatibility complex class I molecules[20].

There are four known inhibitory NK receptor families[21]. The first inhibitory receptor that was discovered was the Ly49 in rodents and it has the ability to bind directly to MHC class I molecules. Another family consists of the killer immunoglobulin (Ig)-like receptors (KIRs) that bind directly to MHC class I. In humans KIRs, comprise a diverse family of receptors with unique structures that are responsible for the recognition of the allelic groups of HLA-A, HLA-B

or HLA-C molecules[22]. The diversity of KIR expression creates a repertoire of NK cells with different MHC specificities[23]. The third receptor is the leukocyte immunoglobulin-like receptor-1 (LIR-1), which when binding to MHC class I molecules diplays an inhibitory signal[24]. The fourth family that can be found in humans and in rodents alike consists of CD94/NKG2 heterodimers.

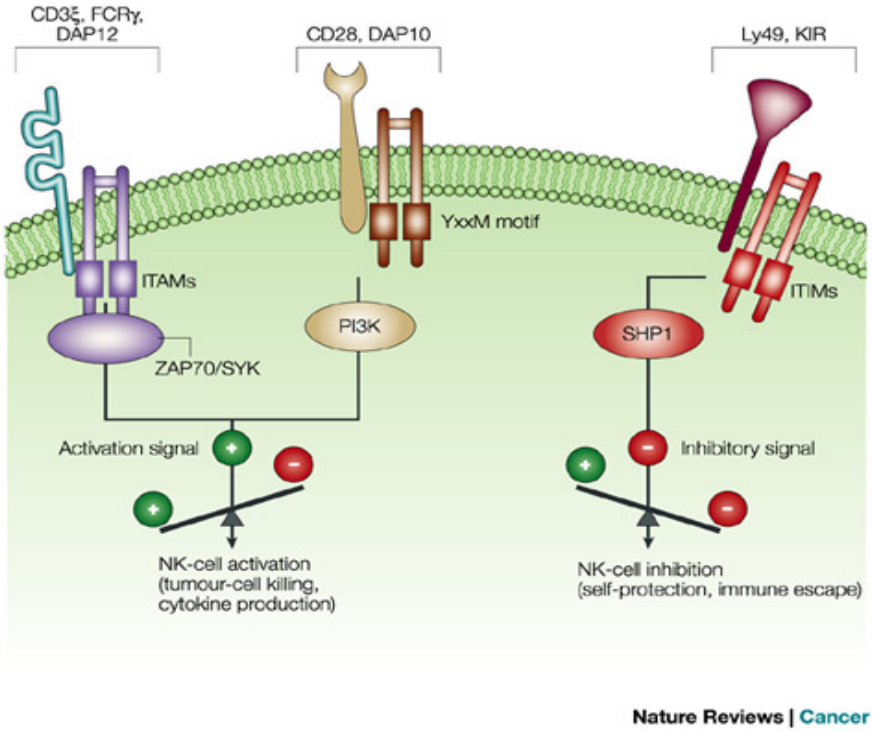

Figure 3. The balancing act of activation and inhibitory receptors is directly responsible for the function of NK cells. Reproduced from Nature Reviews Cancer, Smyth *et al.,* 2002.

4.2.2 Natural killer cell applications

From the 1980's, when lymphocyte-activated killers were firstly used in end-stage patients with solid tumors, till recent days, many advances have been made in the NK cell field[25]. The idea of using NK cells in therapy is promising, especially in the fields of cancer, haematopoietic stem cell transplantation (HSCT) and infectious diseases[26,27]. The application of activated NK cells in haematological patients is currently being explored. One strategy is the enhancement of NK cells' anti-neoplastic activity and the infusion of selected NK cells as an alternative to CTLs for graft versus leukemia (GVL) and thus avoiding GvHD [25].

Hematopoietic stem cell transplantation is a possible treatment for patients with hematologic malignancies[28,29]. The patient is provided with stem cells in order to replace the damaged or destroyed ones after chemotherapy/radiation therapy. The healthy stem cells may be derived either from a donor or from the patient's treated marrow or peripheral blood. The absence of any infection is

critical, during the time that the transplanted hematopoietic stem cell begins to produce white blood cells. However, side effects such as infection, bleeding and graft-versus-host-disease (GvHD) may not be excluded especially in the occasion of an allogeneic donor-received hematopoietic stem cell graft.

One interesting example of cellular therapy closely connected with the above fields, is the NK cell-mediated killing of leukemic cells[30]. This hematopoietic transplantation is based on NK cell alloreactions and it is crucial to improve engraftment, to prevent the GvHD and to reduce toxicity. The prevention of the GvHD, along with the absence of leukemic relapses, is achieved when HLA-C mismatches exist between the donor and the recipient. In fact, these HLA-C mismatches reflect also a difference in the KIR repertoire, which results in that donor NK cells do not always express specific inhibitory receptors to the recipient's HLA-C alleles. Donor NK cells that express for example p.58.1 KIR specific for HLA-Cw2 and HLA-Cw4 alleles, do not match with a recipient expressing HLA-Cw1 and HLA-Cw8 alleles. This way these donor NK cells contribute to the killing of recipient leukemic cells, minimizing the chance of leukemic relapses.

4.2.3 Natural killer cells in hematopoietic stem cell transplantation and GvHD interactions

Currently hematopoietic stem cell transplantation (HSCT) offers the possibility to cure patients with hematological malignancies[31]. Despite the intensive conditioning regimens used, the effect of GvHD develops after transplantation and is often associated with morbidity and mortality. *Ex vivo* marrow graft T cell depletion and post HSCT immunosuppresion are usually not able to prevent graft rejection and leukemic relapses[32]. Working towards the reduction of the incidence of relapses two strategies have been developed; intensified myeloablative conditioning and immunotherapy. In contrast to intensified conditioning that results in increased toxicity[33], immunotherapy has provided some encouraging data particularly in relapse treatment, due to donor lymphocyte infusions (DLI) that lead up to 70% remission[34]. However, as shown from Kolb *et al.*, 1995 there is still a high possibility (41%) to develop GvHD of grade II[35].

Additionally, Asai *et al.*, 1998 have used SCID (severe combined immunodeficient) mice, lacking both T and B cell function, as donors to obtain pure NK cell preparations[36]. This study supports that transplanted NK cells in conjunction with systemic administration of IL-2 promote bone marrow engraftment and have potent anti-tumor effects without inducing GvHD. Due to difficulties in expanding and purifying NK cells in sufficient amounts, clinical studies involving adoptive NK cell transfer have been very limited. Recently, though, a new protocol has been established for large-scale expansion of human NK cells[37], showing that stimulation of donor lymphocytes *in vitro* can lead to a 200-fold expansion after 21 days of culturing. In this study, the term, cytokine-induced natural killer cells (CINK),

will be used for the expanded cell population, since it consist of 55% CD3-$CD56^+$ cells. Based on preliminary data, it such cells may not induce GvHD to the same extent as T cells.

4.2.4 Natutal killer-related cytokines - Interleukin-2 and others

IL-2 is considered to be a NK cell survival factor that is responsible for their survival and enhanced killing activity[38]. IL-2 was the first cytokine to be identified and cloned almost 25 years ago[39]. IL-2 is a multifunctional cytokine. In B cells it stimulates growth and the synthesis of the J-chain and in T cells it stimulates growth -in particular inducing a large number of CD4+ T-cells[40]. Among other hematopoietic cells it stimulates NK cell growth, and induces lymphocyte and NK activity[41]. It also has a role in antigen-induced cell death and it induces antitumor effects. The clinical interest for IL-2 and its receptor is based on the fact that it has a central role for the immune response. Interleukin-2 is a cytokine shown to be released from T cells when they are activated by the presence of a foreign molecule.

The receptor components of the IL-2 family of cytokines are type I cytokine receptors (hematopoietic receptors)[42]. IL-2R can, due to its structural motif, share a receptor component with other cytokines[43,44]. This receptor component is γ_c and is shared by IL-2R, IL-4R, IL-7R, IL-7R and IL-15R. The basis of the fact that γ_c can be shared is that it is a component that can be utilized differentially from one receptor to another depending on the presence of different ligands. However sharing receptor components, like in the case of γ_c, may provide a mechanism through which different cytokines control each other's activities and can allow for the sharing of actions.

Other interleukins such as IL-12, IL-15, IL-18 and IL-21 have also been suggested to contribute to NK cell function[45-49]. In particular IL-12 activates NK cells that secrete IFN-γ, switches T cells to T helper cells (Th1) and inhibits IL-4-induced IgE secretion. IL-12 was initially discovered due to its ability to stimulate interferon-γ production by NK cells and to enhance CD8 cytotoxicity. Similarly, IL-15 induces growth and cytotoxicity of NK cells and it leads to NK cell differentiation. The combination of IL-15 and IL-21 enhances the production of IFN-γ in human NK cells, fact that can be achieved from the synergetic effect of IL-12 and IL-18 as well.

The analysis of cytokine production can be either quantitative or qualitative[50]. For a quantitative investigation, methods such as Northern Blotting, Polymerase Chain Reaction, In Situ Hybridisation, ELISA and Immunofluorescence (flow cytometry, immunohistochemistry) can be used. For qualitative analysis the ELISPOT method, which is similar to ELISA and is based on the analysis of color spots can be used.

4.3 Gene therapy- An overview

Gene therapy in the medical field, normally, implies the cure of an inherited disease by supplying the patient with a correct copy of the defective gene[51]. The two basic approaches to gene therapy are somatic cell therapy and germline therapy. Somatic cell therapy is engaged in the manipulation of ordinary cells that are transfected upon removal from the organism and at a later timepoint injected back in the body, while germline therapy can potentially be used to treat an inherited disease. This thesis is deals with somatic cell therapy. The success of gene therapy is accounted to a large extent to the success of gene transfer. Gene transfer implicates the delivery of a potential therapeutic gene into target cells and different methods for this transfer can be applied e.g. electroporation, gene-gun, or viral vectors. In this thesis only viral gene transfer vectors will be described, with a further emphasis given on retroviral vectors and the model gene of GFP.

4.3.1 Viral gene transfer vectors

Viral vectors offer high gene transfer efficiency both *in vitro* and *in vivo*. The characteristics of the most commonly used vectors are summarized in Table 1. In this thesis, retroviral vectors are used and will be described in further detail.

Vector Group	Genome	Capacity	Persistence	Titre Log_{10} IU/ml	Immunogenicity
Retrovirus	+RNA	8kb	Integrated	6-7	Low
Lentivirus	+RNA	8kb	Integrated	10	Low
Adenovirus	dsDNA	8kb (E3) 28-32 (gutless)	Transient episomal	13	High
Adeno-associated virus	ssDNA	4-5 kb	Integrated Tandem	11	Low
Herpes Virus	dsDNA	30kb	Episomal	8	Low

Table 1. Properties of viral gene transfer vectors

4.3.2 Retroviral vectors

Recombinant retroviruses are commonly used to deliver genes to mammalian cells[52]. Retroviral vectors can be custom-made and they are efficient gene delivery vehicles, because they do not cause any damage during their entrance in the target cells. They consist of a large class of enveloped viruses that contain single-stranded RNA as viral genome. In the nucleus, the retroviral nucleic acid becomes integrated into the chromosomal DNA, a fact that stabilizes the transmission and ensures a long term persistence[52]. The limit of foreign gene sequence that can be packed into a retroviral vector is 8kb. Their advantage is the high transfection efficiency that enables a large proportion of the extracted cells to receive the gene of interest. In this thesis two genes, GFP[53,54] and IL-2[53,55] have been successfully inserted in the genome of both NK cell lines and primary NK cells.

Usually retroviral vectors are obtained from the Moloney murine leukemia virus (MoMLV)[56]. The long terminal repeats (LTRs), the Psi (ψ) and the viral genes are three structural parts of the MoMLV. LTRs are responsible for the initiation and the termination of transcription. The ψ - encapsidation contributes to the packaging of the RNA viral genome into viral particles and the viral genes *gag*, *pol*, *env* encode viral structural proteins, enzymatic proteins and the envelope protein respectively.

Lately there have been many concerns for the clinical safety of retroviral vectors, due to complications over clinical trials. During a trial on the correction of X-linked severe combined immunodeficiency by gene therapy using retroviruses, a retrovirus-mediated gamma gene was transferred into autologous $CD34^+$ bone marrow cells. This retroviral insertion triggered deregulated premalignant cell proliferation[57]. Additionally in a non-primate study, a replication-defective retroviral vector-associated neoplasia was observed in a rhesus macaque, suggesting that currently available retroviral vectors may have long-term side effects[58]. In order to improve safety, prognosis and efficiency, we need to study and classify potential side effects, such as target cell manipulation, transgene insertion, vector expression[59]. Possibly, retroviral vectors that are carrying a 'safety switch', e.g. suicide gene such as thymidine kinase, may be the way to prevent insertional mutagenesis[60].

4.3.3 A model gene - Green fluorescent protein

Green fluorescence protein (GFP) is a very commonly used marker that was originally isolated from the jellyfish *Aequorea Victoria*[61,62]. It can be expressed in either eukaryotic or prokaryotic cells and produces a green fluorescent color when illuminated by blue or UV light. GFP is a useful reporter for monitoring gene expression both *in vivo* and *in situ* without the need for incubation with a second reagent. Detection of GFP can easily be performed in living cells, in tissues, as well as fixed samples [63-65]. GFP has been used throughout our *in vitro* experiments as a positive transduction control, as well as a marker gene for the establishment of a syngeneic model of multiple myeloma through *in vivo* detection of 5T33 GFP^+ myeloma cells.

4.3.4 Gene transfer into NK cell lines and primary NK cells

Stable gene transfer into NK cell lines e.g. the electroporation of the CD18 gene into mutant YT-1 cells[66] and the retrovirally transduction of the chimeric zeta-chain gene into NK3.3 cells was reported already in the mid 1990s[67]. In recent years, there have been several studies that show successful gene transfer into NK cell lines. For example, the IL-2-dependent NK-92 cell line has been transfected with up to 15% efficiency using particle-mediated gene transfer[68] and with up to 20% retroviral transduction efficiency[69]. Similarly, the effect of nucleofection technique using the Amaxa system™[70] on the tested NK3.3 and NKL cell lines was around 8%.

During the last 20 years there has been a series of attempts to transduce primary NK cells, as well, using calcium phosphate coprecipitation, electroporation and liposomal transfection[71,72]. These attempts have been met with mixed success and low efficiency. Recently, there has been some success in transduction of primary NK cells with adenoviral vectors[73], electroporation[74] and hybrid EBV/retroviral vectors[75]. The retroviral transduction of primary PBMCs and NK cells[54,55], which is presented in this thesis, is a complimentary approach to the above techniques.

4.3.5 Cytokine gene-modified cells

Cytokines, which have been widely used as immune modulators, usually work in a paracrine form, after cell-to-cell interaction and the therapeutic levels of systemically applied cytokines are frequently toxic[76-78]. Systemic cytokine delivery should therefore be substituted with a more localized method of cytokine release. An alternative approach for application of cytokines to stimulate immunocompetent cells could be the production of auto stimulated cell lines expressing the gene of the cytokine of interest.

The cytokine of interest can be successfully expressed from the target cells (REF), however the non-localized delivery remains a critical issue, since it leads to certain side effects and the activation of neighboring cell populations[79,80].

The concept of cytokines expressed in a strictly localized and confined space has been around for some time now, with attempts to induce endogenous cytoplasmic IL-3[81] and to target chemokines intracellularly in the ER[82]. However not until recently, was it shown that cytokines can be targeted to particular intracellular regions using specific localization signal peptides[83]. A stable and active form of GM-CSF was successfully target into the subcellular compartments of the cytoplasm, the endoplasmic reticulum and the nucleus[84].

Our approach here, was to express intracellular forms of IL-2 into NK cells[53]. Our aim was to generate IL-2 independent NK cells, but that expressed IL-2 into two ways; one naturally and the other in the confined to the endoplasmic reticulum.

4.4 Multiple myeloma – An overview

One hematological malignancy that is a potential candidate for immunotherapy is multiple myeloma (MM)[85,86]. MM is a B cell malignancy that is characterized by the accumulation of malignant plasma cells within the bone marrow, the presence of a monoclonal immunoglobulin in the serum or urine and the development of osteolytic bone lesions. In general, plasma cells are produced by stem cells in the BM. When foreign substances enter the body, B cells develop into plasma cells to help fight infection and disease. In the case of

MM, a genetic damage to a developing B cell transforms it into malignant plasma cell. These cells become abundant in the BM, leading to soft spots (osteolytic lesions) in the bone. These regions weaken the bone, causing pain and increasing the risk of fractures. Although it affects the bone, MM is considered to be a hematologic cancer, since it is transported through the blood stream. MM accounts for 20% of all hematological malignancies deaths.

Bone destruction by osteolytic lesions is caused by two separate events; firstly the rapid growth of myeloma cells pushes aside normal bone-forming cells resulting in bone damaging, and secondly the production of substances that activate osteoclasts (bone-resorbing cells) is increased. Normally osteoclasts break down old or worn out bone and work with osteoblasts (bone-forming cells) to repair the bones. In MM, the increased activity of osteoclasts causes osteoporosis (bone loss). Moreover, the condition of hypercalcemia becomes apparent in cases where the level of calcium in the bloodstream rises due to bone destruction. Due to myeloma cell growth the production of normal blood cells is impaired. The production of the M protein that is produced by MM cells can thicken the blood. Meanwhile, the reduction of white and red blood cells, as well as of platelets, can result in increased infection risk, anemia and prevention of blood clotting respectively. Finally, the secretion of the M protein results in kidney circulation problems, as well, reducing calcium excretion and increasing urine production and the chance of dehydration.

Up to this date, no cause of MM is identified. The immune system, genetic factors, chemicals and exposure to radiation are thought to be associated with MM.

4.4.1 Multiple myeloma treatments

MM treatment focuses mainly on suppression and disease containment. After MM diagnosis, an initial therapy includes regimens such as dexamethasone with or without thalidomide and VAD, a cocktail of vincristine, adriamycin and dexamethasone. In a latter stage, if patients show disease progress, chemotherapy and autologous stem cell transplantation (ASCT) is recommended. Despite of the advances in ASCT with high melphalan doses and chemotherapy treatment[87,88], MM still remains largely an incurable disease[89]. Therefore a more effective treatment is warranted.

4.4.2 Multiple myeloma mouse models

For the better understanding of MM, it is of great importance to monitor MM cells during the progression of the disease. Up to now there have been some studies where myeloma cell lines [90,91]and BM cells from myeloma patients were injected in to SCID mice[92,93]. Furthermore, NOD/SCID and C57BL/KaLwRij mouse strains have been used as experimental models[94]. However it was not until recently that a study using NOD/SCID mice aimed in detecting myeloma cells in real time by marking them with GFP[95]. Although these data showed the anatomical distribution and pathophysiological

manifestations of developed MM, the immunodeficient nature of NOD/SCID mice ignores the role of the immune system in the development and treatment of MM. Additionally, it has been suggested that the immunocompetent C57BL/KaLwRij mouse strain is a favorable model due to spontaneous development of MM[96]. Following these lines, we transduced 5T33MM cells with GFP and injected them intravenously into C57BL/KaLwRij mice[97].

4.4.3 Natural killer cells in multiple myeloma

NK cells, as previously mentioned, do not require preactivation in order to recognize and kill tumor and infected cells. Myeloma cells are susceptible to NK cell lysis[98] and during MM, the number and the state of activation of NK cells increases. NK cells have an innate anti-MM cytotoxic activity. Drugs, such as thalidomide may further augment this effect[99,100].

Furthermore, IL-2 activated lymphocytes have been shown to have a potential antitumor efficacy against autologous and allogeneic human myeloma cells[101]. Additional clinical IL-2 studies indicated that plasma cells are sensitive to LAK cell lysis and moreover that IL-2 infusions result in a substantial increase in TNF and IFN-g production[102]. However, none of the above studies examined which cells were capable of exerting an autologous anti-myeloma activity after cytokine induction. Our group has shown that IL-2 activated NK cells are the major effector cells responsible for this antitumor activity[103].

5. AIMS OF THE PRESENT STUDY

The long-term objective of this study is to generate natural killer (NK) cells to be used for clinical therapy, without the need of exogenous interleukin 2 (IL-2) administration. The aim was also to test the possible effect of IL-2 activated NK cell in an experimental multiple myeloma (MM) model.

The specific aims leading step-by-step to our main goal are listed below:

I) To genetically modify the IL-2-dependent NK-92 cell line, to express IL-2 in a localized and controlled manner

II) To establish an experimental protocol for retroviral gene transfer into primary human NK cells, using a green fluorescent protein (GFP) vector and to initiate studies aiming at expressing IL-2 in $CD56^{+}CD3^{-}$ PBMCs, in order to obtain NK cells independent of exogenous IL-2 for survival and function.

III) To establish a murine multiple myeloma model using GFP MM cells

IV) To identify the effector cells in peripheral blood that are capable of autologous cell killing in a myeloma model and to use these cells for adoptive immunotherapy

6. METHODOLOGY

6.1 Plasmids – Retroviral vector coding for IL-2 and other control genes

Three transgenes expressing IL-2 were designed in Paper I. The first transgene was targeted to the IL-2 subcellular receptor alpha, the second to the cytoplasm and the third to the endoplasmic reticulum. The IL-2 variants were cloned using PCR. The PCR products were later cloned into pCR4BluntTOPO vector. Clones were characterized by using restriction analysis and cycle sequencing. In paper I of this thesis, the emphasis was limited to two of the three original constructs; one expressing wild type IL-2 (secreted – IL-2WT) and one targeted to the ER (IL-2ER).

The green fluorescent protein (GFP) gene was used as a positive control in transduction experiments in papers I, II, III and for sorting purposes in paper IV. The red fluorescent protein (RFP) was used for control and sorting purposes in paper IV. The plasmid pMO3TIN, containing the herpes simplex virus thymidine kinase (HSV-*tk*) and NeoR genes linked by poliovirus IRES in the MoMLV backbone, was used for selection purposes in paper III.

6.2 Transfection and retroviral transduction of cell lines and PBMCs

Phoenix GP cells were transiently transfected with 3μg of vector construct plasmid (IL-2WT, IL-2ER, GFP and RFP) and 1μg pMD-G (encoding vesicular stomatitis virus envelope glycoprotein) per 35 mm cell culture well, using the Fugene 6 reagent according to manufacturer's instructions in papers I-IV. Virus supernatants were collected from transfected PhoenixGP cells 24 and 48 hours after transfection, filtered through 0.45μm Millex-GP syringe-top filter and used immediately for transduction. The obtained virus titers varied up to 10-fold (10^5-10^6 virus particles/ml). The vector-containing supernatants were used to transduce different cell types, such as NK-92 (Paper I), 5T33MM (Paper III) and $DX5^+$ NK mouse cells (Paper IV), by centrifugation at 1000xg for one hour in the presence of 4μg/ml polybrene, or by different modifications thereof (see below).

All PBMCs (Paper II) were transduced, on day 5 of culture, with PG13-collected supernatant either by a 2 hour-centrifugation at 1000g in the presence of 4μg/ml polybrene and 500IU/ml IL-2. After 48 hours the cells were washed to remove polybrene, and divided in groups for further culturing and analysis. As a control, PBMCs of the same donor were grown and mock transduced under similar conditions as transduced cells.

In all cases viral titers were estimated indirectly by transducing HeLa cells with different volumes of retroviral-containing supernatant. Increasing volumes of supernatant (2, 20 and 200 ml) were plotted against the percentages of fluorescent target cells determined after 48 hours by FACS analysis. Titers were calculated according to the following formula: Titer (HeLa-transducing

units/ml) = (number of HeLa cells infected) x (% of positive cells/100)/volume of supernatant (in ml). In the case of IL-2 positive cells, cells were stained with intracellularly with anti-IL-2, prior to FACS sorting.

6.3 *Ex vivo* expansion of PBMCs and subpopulations thereof

The *ex vivo* culture and expansion of PBMCs was performed as described in more detail in papers II. Briefly, buffy-coat preparations from healthy blood donors were obtained from the blood bank at Karolinska University Hospital Huddinge, Stockholm, Sweden. They were isolated by gradient centrifugation, using Lymphoprep. After washing twice with PBS, cell viability was assessed by trypan blue dye exclusion and the cells were plated onto six-well dishes at 0.5 X 10^6 cells/ml. CellGro medium was used in all the cultures with the addition of 10 ng/ml OKT-3. OKT-3, an anti-CD3 antibody, stimulates T cells, whose cytokine and growth factor secretion seems to promote NK cell growth. On day 5 of expansion, OKT-3 was washed out and PBMCs were cultured there after in CellGro medium supplemented with 5% HS and 500 IU/ml IL-2. The culture medium was replaced every 1-2 days until the end of culture.

6.4 *Ex vivo* separation of mouse cells

In order to obtain mouse NK cells from C57Bl/KaLwRij mouse single-cell suspensions (Paper IV), the DX5+ Mouse Microbeads kit (Miltenyi Biotech Inc., CA, USA), was used according to manufacturer's instructions.

6.5 Flow cytometric analysis

The phenotype of the cell lines, PBMCs and single cells suspensions from different mouse organs (spleen, liver, BM, and thymus) were analyzed using a FACSCalibur flow sorter (Papers I-IV). Four-color fluorescence was used according to standard procedures. Cell were mixed with appropriate concentrations of different dye-conjugated monoclonal antibodies, incubated for at least 15 minutes at room temperature and finally washed with PBS prior to flow analysis. The most frequently used antibodies were the CD56APC, the CD3PerCp and the anti-IL-2PE, all purchased from BD Pharmingen, CA, USA. NK cells were defined as $CD56^+CD3^-$, T cells as $CD56^-CD3^+$, and both NK and NKT as $CD56^+$. Propidium iodide (PI) staining was used for dead cell exclusion. In each sample, at least 10.000 cells were acquired in the analysis region of viable cells, defined by side and forward scatter.

6.6 Cytotoxicity Assay

The cytotoxic function of NK and/or T cells as effector cells was measured by a standard 4 hour ^{51}Cr-release assay during different timepoints of culturing. As targets, the NK-sensitive K562 cell line (Papers I-II) and in Paper IV the 5T33MM, YAC-1 cell lines and fresh autologous cells, were used. Target cells were labeled with 100µl ^{51}Cr and incubated for one hour at 37^{o}C. Effector cells were counted using trypan blue dye exclusion and mixed with target cells to obtain an effector: target ratio of 10:1, 3:1, 1:1 and 0.3:1. Culture medium was used as a negative control, and for a positive control cells were incubated with

1% Triton X. After incubation in a V-bottom shaped 96-well plate for 4 hours at 37°C, 70µl of each supernatant was aspirated from each well and counted in a gamma counter. The percentage of spontaneous release was calculated from the following formula: % specific ^{51}Cr release = (sample release-spontaneous release) / (maximum release – spontaneous release) x 100.

6.7 Immunostaining of IL-2 modified cells

To verify the expression of IL-2 in the endoplasmic reticulum area, IL-2-modified Cos-7 cells were co-stained with an anti-human IL-2 antibody, as well as with an ER-marker, in order to verify the location of IL-2 expression (Paper I). During this process, initially, the Cos-7 cells were fixed, permeabilised and incubated with a blocking buffer containing goat serum. Subsequently, cells were incubated with a mix of primary purified rat anti-human IL-2 antibody and a rabbit anti-calreticulin ER-Marker. After washing out this mix, cells were incubated with a mix of secondary goat anti-rat IgG antibody and a goat polyclonal antibody to rabbit IgG. Finally, stained cells were rinsed and counterstained with propidium iodide, prior to visualization by a fluorescent microscope.

6.8 ELISA

For the quantitative determination of human IL-2 (Paper I), mouse IL-6 (Paper III) and mouse IL-2 (Paper IV), the OptEIA Human IL-2 ELISA Kit II, the murine IL-6 and IL-2 BD OPTEIA ELISA Kit, respectively, were used according to the manufacturer's (BD Biosciences, CA, USA) instructions. The interleukin levels were determined by comparing the optical density results to standard curve using recombinant IL-2 or IL-6 provided by the manufacturer.

The levels of IgG2b in the cell culture supernatants were measured by a sandwich enzyme-linked immunoabsorbent assay (ELISA) (Paper III). Micro-ELISA plates were coated overnight at 4°C with goat anti-mouse IgGb in carbonate buffer pH 9.8. The plates were then blocked for 2 hours at room temperature with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). After blocking, the samples or standard IgG2b in serial dilutions with PBS containing 1% Tween-20 were added, and the plates were incubated overnight at 4°C. Thereafter, the plates were washed three times with PBS containing 1% Tween-20. For IgG2b measurement, the plates were incubated goat anti-mouse IgG2b for 2 hours at RT. Finally, all the plates were developed. After 20 to 30 minutes of incubation at RT, the color reaction was quantified by an ELISA reader at 405 nm.

6.9 Mice and cell lines in the multiple myeloma study

Female and male C57BL/KaLwRij mice were purchased from Harlan CPB (Horst, The Netherlands) and kept in our animal facilities at the Clinical Research Centre at Huddinge University Hospital, Karolinska Institutet for studies in paper III and IV. They were housed under conventional conditions including access to tap water and standard chow ad libitum. All mice were 8 to

10 weeks old at the beginning of each experiment. Experiments performed in this study were approved by the local ethical committee in South Stockholm, Sweden.

6.10 Adoptive transfer of natural killer cells

Transduced and control NK cells were injected i.v. at established doses in C57Bl/KaLwRij mice that had been previously inoculated with 5T33MM cells (Paper IV). Mice were then monitored for survival and flow cytometric analysis was performed.

7. RESULTS

7.1 Targeting interleukin-2 to IL-2-dependent NK-92 cells (Paper I)

The IL-2-dependent NK-92 cell line was genetically modified by the introduction of the IL-2 gene in two different ways, in order to eliminate the need of exogenous IL-2 administration. As a positive control the GFP gene was used. The two forms of IL-2 that were expressed in NK-92 cells differed in that one generated secreted IL-2 (IL-2WT) and the other one generated endoplasmic reticulum-resident IL-2 (IL-2ER). Both forms of IL-2 sufficed to promote growth and survival of the IL-2-dependent NK-92 cells.

The extent of paracrine and autocrine growth stimulation was also assessed. More specifically, mixed populations of modified and non-IL-2-modified NK-92 cells, demonstrated that ER-targeted IL-2 did not support the growth of neighboring NK-92 non-IL-2-modified cells, and indicated that IL-2 does not leak to the supernatant (endoplasmic reticulum targeting). In contrast, the IL-2WT form was secreted in a natural way and supported the growth of neighboring cells. These data were also confirmed by determination of the IL-2 concentration levels in cell culture supernatant by ELISA. Both types of IL-2-transduced NK-92 cell lines showed comparable functional activity and cytotoxicity to parental NK-92 cells.

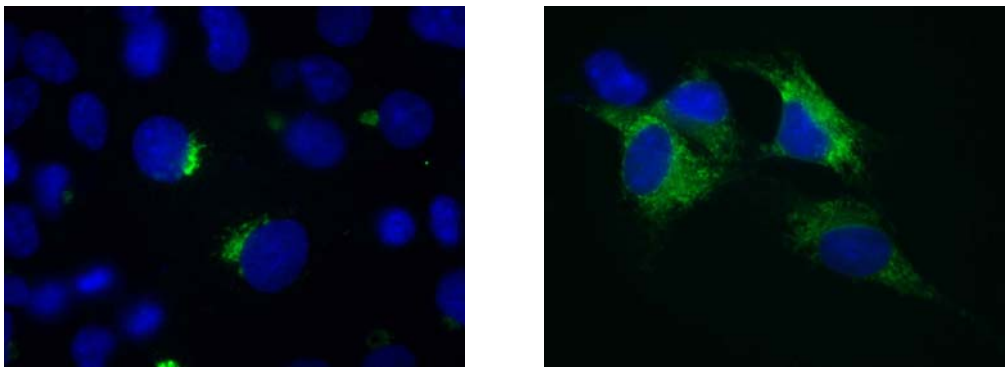

Figure 4. NK-92 cells transduced with IL-2WT (left panel) and IL-2ER (right panel).

The data obtained in Paper I demonstrate the ability of ER-retained IL-2 to provide autocrine growth stimulation to NK-92 cells, without the secretion of the cytokine to the extracellular compartment. Hence, therapy with IL-2ER gene-modified autoactivating NK cells should potentially avoid side effects imposed by exogenously administered IL-2.

7.2 Green fluorescent protein gene (GFP) transfer into primary human natural killer cells (Paper II)

The efficiency of retroviral transduction, into primary human NK cells, was assessed after the transduction of PBMCs with a GFP vector. Consequently,

PBMCs from healthy donors were expanded *ex vivo* for a period of 21 days. Retroviral transductions were carried out by replacing the culture media with retrovirus-containing supernatants during 2-hour incubations on days 3, 4, 5, 6, 10, 15, or 20 (single transduction) or days 5 and 6 (double transduction). The optimal time for retroviral transduction was determined to be day 5.

NK ($CD56^+CD3^-$) cells showed a median of 27.2% transduction efficiency after a single transduction (transduction on day 5) and a median of 47.1% transduction efficiency after two transductions (transduction on days 5 and 6), 24 hours after exposure to retrovirus-containing supernatants. On day 21 after initial culture, 51.9% of NK cells were transduced after one transduction round (transduction on day 5) and 75.4% after two transductions (transduction on days 5 and 6).

During this study, it was thus successfully shown that primary NK cells can be transduced with GFP, without any detectable changes in phenotype, such as receptor expression or cytotoxic function. This may open up new possibilities in the studies of NK cell biology and the development of NK cells for immunotherapy regimens.

In preliminary studies, IL-2WT and IL-2ER constructs (from Paper I) were used to transduce PBMCs cells, in order to produce IL-2-independent primary NK cells. PBMCs from healthy donors were expanded and transduced on day 5 by pre-coating, using IL-2 or GFP retroviral supernatants. Forty eight hours later, in order to assess if IL-2-transduced PBMCs were independent of IL-2 administration, the cells were either washed twice to remove exogenous IL-2, or continuously kept with exogenous IL-2 added in the culture medium. All groups of PBMCs showed comparative proliferative capacity during the first ten days of expansion. However, IL-2-transduced PBMCs, showed higher survival at all observation times in the absence of exogenous IL-2, in comparison with non-IL-2-transduced cultures. The percentage of $CD56^+CD3^-$ cells increased within the bulk PBMC population, irrespectively of exogenous IL-2 administration, however the absolute $CD56^+CD3^-$ cell number increased only in the presence of exogenous IL-2. The $CD56^+CD3^-IL\text{-}2^+$ cell population continued to expand after removal of exogenous IL-2 only in PBMC cultures transduced with IL-2ER (and not when transduced with IL-2WT), despite the fact that the total absolute $CD56^+CD3^-$ cell numbers decreased. These results indicate that $CD56^+CD3^-IL\text{-}2ER^+$ cells are capable of proliferation even in the absence of exogenous IL-2. These studies are currently in progress.

7.3 Establishment of a GFP multiple myeloma mouse model (Paper III)

In order to establish a mouse model, where the MM cells could be monitored *in vivo*, 5T33MM cells were retrovirally transduced with GFP and injected intravenously into C57Bl/KaLwRij mice. In addition, 5T33MM cells were transduced with herpes simplex virus thymidine kinase (HSV-tk) as a control.

Functional characteristics of GFP transduced and non-transduced cells were compared *in vitro* as well. After inoculation of GFP positive 5T33MM in C57Bl/KaLwRij mice, the survival kinetics and distribution of GFP positive cells in tissues were evaluated. Transduced and non-transduced 5T33MM cells exhibited a similar growth rate and produced comparable IgG2b and interleukin-6 levels as assayed by ELISA. Injection intravenously of both non-transduced and GFP-transduced MM cells to C57BL/KaLwRij mice resulted in paraplegia. At the time of paraplegia, a substantial number of GFP-transduced MM cells were detected in the BM, the spleen, and the liver, and to a lesser extent in the lymph nodes. The BM of paraplegic mice contained higher numbers of GFP-transduced MM cells compared to that of non-paraplegic mice.

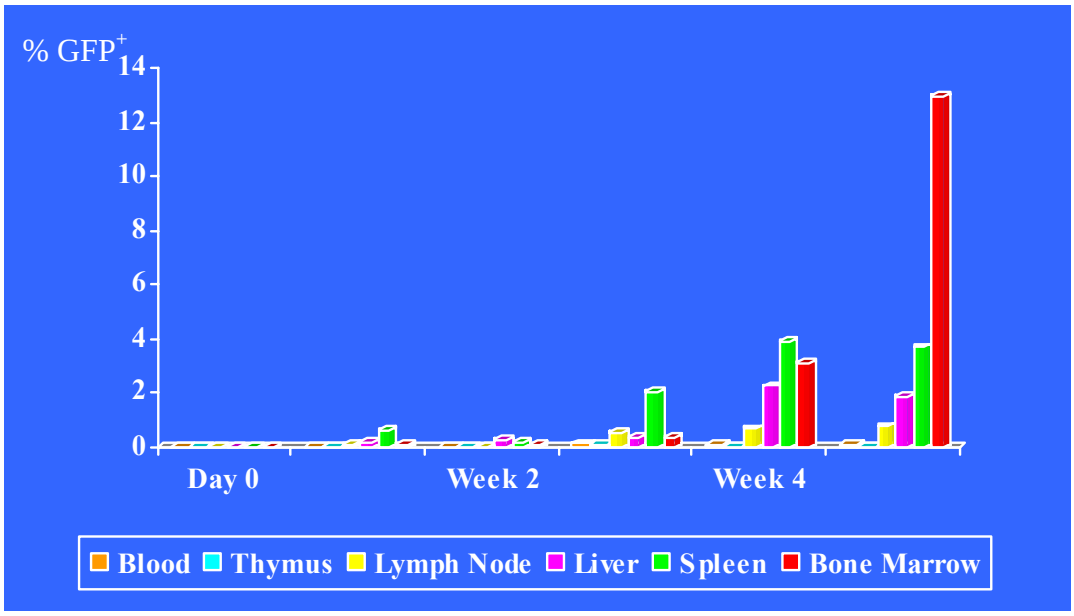

Figure 5; Biodistribution of myeloma cels by ex vivo FACS analysis

This study demonstrates that MM cells can be easily traced in an immunocompetent host, when injected MM cells are GFP-transduced. This model simplifies the analysis of homing pattern studies and the evaluation of therapeutic effects of various treatment approaches.

7.4 The protective role of IL-2-activated natural killer cells in a multiple myeloma model (Paper IV)

In this study, the cytotoxic effects of IL-2 activated NK cells and cytotoxic T-cells (CTLs) towards 5T33MM cells were compared *in vitro* and *in vivo.*

In vitro, NK cells and cytotoxic T lymphocytes (CTLs), originated from single-cell suspensions (spleen, liver, thymus, lymph nodes and BM) of C57BL/KaLwRij mice previously immunized with irradiated 5T33MM cells. NK cells obtained using CD49b ($DX5^+$) magnetic microbeads (positive fraction) and T cells (negative fraction) were compared in co-cultures with

irradiated 5T33MM cells for 5 days with or without IL-2. Using a ^{51}Cr release cytotoxic assay, we could demonstrate that IL-2 activated NK cells display a significantly higher killing efficacy than IL-2 activated CTLs against syngeneic 5T33MM tumor cells.

Anti-NK1.1 and anti-CD8 monoclonal antibodies were used for *in vivo* depletion. Retroviral transduction of the marker gene DsRed2 into NK cells was tested for its tracking feasibility in an adoptive transfer setting. *In vivo*, following IL-2 administration, mice that did not undergo any effector depletion showed the longest survival, followed by, in decreasing order, CD8-depleted and NK-depleted ones, indicating that NK cells were the major effectors exerting autologous tumor clearance after cytokine induction. Gene transfer of RFP into NK cells was successful and it did not have any side effect during our *in vivo* studies. Moreover, the adoptive transfer of gene modified and unmodified IL-2 activated NK cells showed a significant delay in tumor development.

IL-2 activated NK cells efficiently killed 5T33MM cells *in vitro*, and were also the main effector cells in myeloma studies *in vivo*. This study highlights the potential beneficial use of IL-2-induced NK cells after adoptive transfer in an immunocompetent mouse model. Such cells could potentially result in elimination of autologous myeloma cells and prolonged mice survival.

8. DISCUSSION

NK-92 cells transduced with either IL-2WT or IL-2ER (the latter confined IL-2 expression in the endoplasmic reticulum) were compared for their ability to proliferate and survive without exogenous IL-2. Both constructs could be successfully transduced into NK-92 cells, enabling them to proliferate and survive without exogenous IL-2 administration. Our results with IL2WT transduced NK-92 cells were similar to those obtained by Nagashima et al, 1998[69], who attempted to deliver the IL-2 gene into the NK-92 cells. However, there was no effort of targeting IL-2 into specific subcellular compartments in that study. In our report we could show that by targeting IL-2 into the confined area of the ER, IL-2 did not leak to the supernatant and could thus not support the growth of non-IL-2 transduced cells. Our results acted as the first proof of principle that it is possible to express IL-2 intracellularly. This way it is possible to avoid the toxic effects of systemic IL-2 administration and the activation of neighboring cell populations, e.g., in the treatment of a relapsing hematological tumor after hematopoietic stem cell transplantation[30]. In the case of the activation of neighboring cells, e.g. T cell activation leads to GvHD[104] and in case of Treg overstimulation to severe immunosuppresion[79].

Following the above findings, we decided to move on to primary NK cells and examine the possibility of transducing primary NK cells and in general PBMCs. GFP was initially employed to transduce primary NK cells. GFP is

generally a stably expressed protein, and can easily be visualized under a UV microscope and detected during flow cytometric analysis. PBMCs were acquired from healthy donor buffy coats and expanded for 21 days. $CD56^+CD3^-$ cells within PBMCs were successfully transduced with GFP on day 5 of culture and until day 21 the majority of the transduced cells had a $CD56^+CD3^-$ phenotype. Furthermore, gene transfer did not show to affect the survival, proliferation and phenotype of the GFP-transduced cells. These results were similar to that of a recent study by Becknell *et al.*, 2005, who could also demonstrate that it was possible to infect primary NK cells and NK cell lines with GFP and other genes of interest using an EBV/retroviral hybrid vector [75]. Our second study proved that it was possible to successfully transduce NK cells with a retroviral vector and opened the way for further experimentation with therapeutic genes, such as IL-2.

A further step in this study would be the retroviral transduction of $CD56^+CD3^-$ PBMCs with the IL-2WT and IL-2ER constructs, in order to produce IL-2-independent NK cells. Such cells could have an immunotherapeutical potential. For example, patients may benefit from the infusion of autologous NK cells, which have been obtained by a blood sample, *ex vivo*, isolated from the other PBMCs, IL-2 modified and expanded (Figure 6). At the same time, such a patient would avoid the process of systemic IL-2 administration with its undesired side effects. These IL-2-independent NK cells could, for safety reasons, be further transduced with vectors carrying a suicide gene, e.g. thymidine kinase. Such cells will need to be expanded into large numbers before infusion back to patients, but most importantly it is of major importance to test their therapeutical potential experimentally, e.g. in a mouse model.

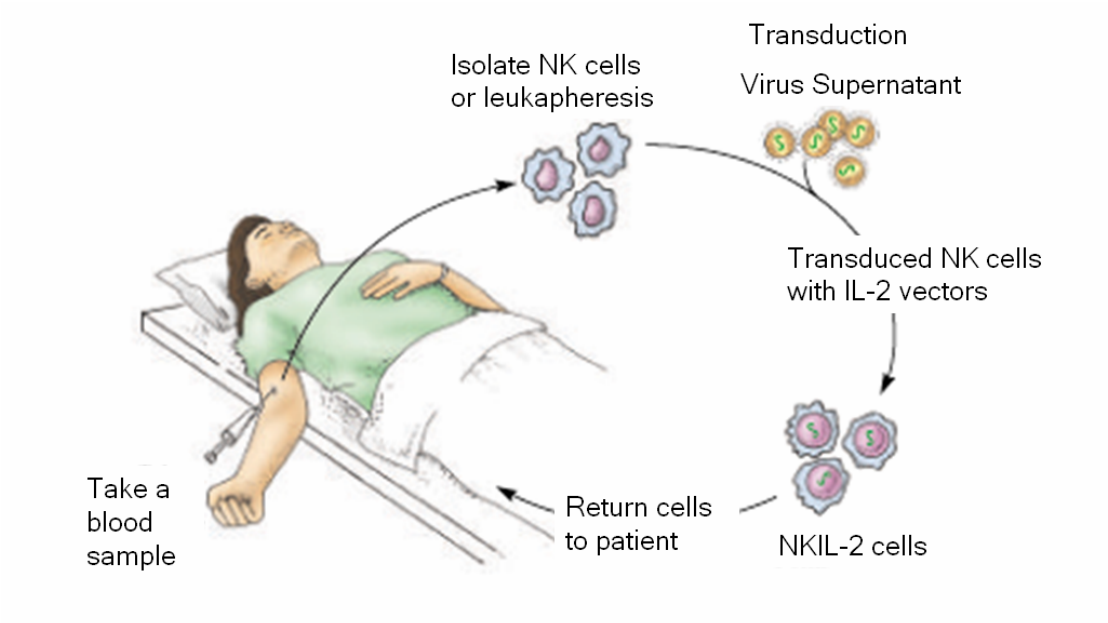

Figure 6. Schematic diagram of a potential gene modified NK cell clinical trial

In parallel, to our studies on the transduction of IL-2 and GFP into NK cells, in order to obtain an *in vivo* model, and due to our prior experience in the MM field, we investigated the possibility to monitor multiple myeloma cells *in vivo*. For this purpose 5T33MM cells were transduced with GFP and injected into the immunocompetent C57Bl/KaLwRij mice. As a result, it was not only possible to detect and localize the myeloma cells *in vivo* during the course of the disease, but also to assess the behavior of retroviral transduced cells in a mouse model.

This GFP multiple myeloma mouse model and the potential of using IL-2 transduced cells for immunotherapy, spurred the investigation of the effect of cytokine induced NK cells in a MM setting. The 5T33MM cell line was inoculated into C57Bl/KaLwRij mice and it was shown that exogenously IL-2-activated NK cells can efficiently eliminate these syngeneic MM cells and prolong the survival of the mice. These findings are in line with reports that show lymphokine activated NK cells could increase the response to in renal cell carcinoma and relapse melanomas cases[105], and the autologous activity in hematological malignancies[106,107]. However there are also studies that present $CD8^+$ T cells as responsible for autologous tumor metastatic melanoma clearance[108]. Additionally, the transduction of NK cells with RFP and 5T33MM cells with GFP enables as to monitor the disease development and to study the importance of their co-localization.

A future perspective of this project could be to transduce NK cells derived from mice, with the above mentioned human IL-2 constructs, which should then be tested for their efficiency in a mouse setting, or to use similar murine IL-2 constructs. This way it will be possible to compare the effects of exogenously IL-2-activated NK cells with the IL-2ER transduced NK cells against MM.

9. CONCLUSIONS

Our conclusions, step by step are listed below.

- An autocrine growth stimulation, without IL-2 secretion to the extracellular compartment, could be obtained in the cell line NK-92 after retroviral transduction with ER-retained IL-2 (IL-2ER). (Paper I)

- Primary human NK cells (CD56+/CD3-) could be transduced and express green fluorescent protein (GFP) without detectable alterations in phenotype or function. (Paper II)

- Retrovirally GFP-transduced 5T33MM cells could easily be traced in an immunocompetent C57Bl/KaLwRij mouse model. (Paper III)

- IL-2 activated NK cells were responsible for myeloma cell killing and increased survival of C57Bl/KaLwRij mice. (Paper IV)

10. ACKNOWLEDGEMENTS

I would like to thank my past and present colleagues, friends and family, who have supported and contributed to this work over the past years. In particular, I would like to express my gratitude to;

Associate Professor **M. Sirac Dilber**, my *supervisor*, for accepting me in his research group, for providing me all the financial means for this project and for always highlighting the importance that you should be excellent in selling your idea.

Professor **Hans-Gustaf Ljunggren**, my *co-supervisor*, for sharing his scientific expertise, especially in the field of innate immunity and for always being positive, supportive and enthusiastic about my work.

Dr **Alar Aints**, my *co-supervisor*, for spending long hours together in the lab, especially during the laborious molecular biology stages of this project during the first years.

Professors **Tina Dalianis** and **Lars Ahlund-Richter**, my *friends*, for always finding some spare time in their already busy schedule to accommodate my inquiries and requests. For always being so positive and supportive, even when everything was going wrong, and trying your absolute best to cheer me up (that was a tough job!).

Professor **Gösta Gahrton**, for being so interested in our work, helpful with the editing of our papers and always bringing new suggestions in our work.

Professor **Birger Christensson**, Pathology Department, for help with the core facilities in his laboratory and critical reading of our manuscripts.

Professor **Moustapha Hassan**, Head of the Hematology Laboratory, for always being around with a cheerful attitude and ready to help me with everyday problems. Many thanks for assisting me with the Hematology ward.

Professor **Jan Bolinder** and Professor **Jan Palmblad**, present and past Heads of the Department of Medicine, Professor **Per Ljungman**, Chairman of the Division of Hematology for providing a pleasant and creative working atmosphere.

All the past and present members of my group; **Mari Gilljam** and **Birgitta Stellan** for always being around and kind to help with whatever the problem was, Dr **Hayrettin Guven** for being such a great culture lab partner and always pleased to offer a second hand, Dr **Jose Arteaga** for all the advising with experimental design and flow charts, **Evren Alici** for being always around to help with literally any issue, **Alexandra Treschow**, for being so cheerful and

positive (all the best to your new venture), **Christian Unger** for always being so energetic and the heart of great social events, Dr **Manuchehr Abedi-Valugerdi** for sharing your immunology and animal expertise and your critical comments on experimental design, **Ulrika Felldin** for always being keen to provide an extra hand of help whenever needed in the lab, **Hernan Concha** for always sharing a 'Kalimera' with me, for being an open historical and political book, for sharing with me his passion for Greek history and for spending long hours in the lab teaching me flow cytometry, Dr **Fernando Guimaraes**, for bringing the warmth and kindness of Brazil in the group (one day I will make it to Sao Paolo), for introducing me to the in vivo world and for appreciating the meaning of 'Good Morning', **Daniela Liebrenz** and **Tolga Sutlu** for molecular work discussions.

My colleagues in the *Avaris* office; **Thomas Bjarke** and **Hans-Peter Ekre**, CEOs of Avaris AB, for providing a quite and comfortable working environment, Dr **Elizabeth Tornquist** for being responsible of management and organization during the first years, **Iulian Opera, Joanna Viola, Maroof Hassan and Beatrice Sjöberg** for all discussions.

All my friends at the *Hematology Lab*, **Gayane Avetisyan** for all the nice moments during conferences, Dr **Marina Vita,** Dr **Ramin Tehranchi**, **Rasheed Zaki Khan, Parvaneh Afsharian**, Dr **Jan Schmidt-Mende**, **Monika Jansson**, **Lalla Forsblom**, **Sofia Bengtzén**, **Anna Wallblom**, **Kristina Friberg**, **Christina Nilsson**, **Emma Emmanuelsson**, Dr **Zuzanna Hassan, Ann Svensson**, **Johana Forsman**, **Kerstin Jönsson-Videsäter and Birgitta Åhnsen** for creating a friendly working atmosphere.

All members of *Edvard Smith's group*, Professor **Ted Smith**, Dr **Abdalla Mohamed Jama** and Dr **Beston Nore** for sharing with me their vast knowledge in molecular biology and cell culturing**,** Dr **Magnus Bäckesjö**, Dr **Anna Berglöf**, Dr **Leonardo Vargas**, Dr **Jessica Lindvall**, **Lotta Asplund**, Dr **Karin Lundin**, **Mattias Svahn**, **Oscar Simonsen**, **Juhanna Heinonen**, **Emelie Blomberg, Ge Rongbin** and **Liang Yu** for always being kind to help with reagents and everyday laboratory issues.

All my friends at *CIM*; **Ann Hellström** for always being so kind and helpful, **Yenan Bryceson** and Dr **Daria Donati** for fun time during conferences in Greece, Hawaii, St Petersburg and Swedish archipelago, Dr **Maire Quigley** for always suggesting me a better way to do things at work, Dr **Karl Malmberg** for inspiring scientific discussions, **Anette Hofmann** for help with the confocal microscope, Dr **Adnane Achour**, **Annelie Tjernlund**, Dr **Benedict Chambers,** Dr **Mayte Bejarano**, Dr **Mark Wareing**, Dr **Jakob Michealsson**, Dr **Bettina Baumann**, Dr **Antonio Barragan**, Dr **Steve Applequist**, Dr **Alf Grandien**, **Mikolaj Malinowski**, Dr **Rickard Glas**, **Hong Xu**, Dr **Anna Smed Sörensen**, **Arina Samarina**, Dr **Gail Mullins**, Dr **Markus Moll**, **Cattis Persson**, Dr **Mattias Svensson**, **Niklas Björkström**, **Mattias Carlsten** , Dr

Thorbald van Hall, Dr **Linda Johansson**, **Lena Radler**, **Monica Hultcrantz**, **Michael Hűhn**, **Pontus Thulin, Claudia Thilo**, Dr **Shirin Heidari** and **Emma Persson**, Dr **Anna-Lena Spetz**, Dr **Johan Sandberg**, Dr **Niclas Hitziger**, **Veronica Gonzalez**, Professor **Johan Frostegård**, Dr **Malin Flodström**, **Stella Jakobsson** and **Henrik Lambert** for creating and contributing to an excellent scientific environment at F59 in Karolinska Hospital in Huddinge.

Dr **Ennio Carbone**, Dr **Petter Höglund** and Dr **Louise Berg** for organising an excellent international PhD course on tumour biology and innate immunity in *Catanzaro*, Italy. Many thanks to all the participants for inspiring conversations, especially the KI participants; **Anna De Geer**, **Fang Zong**, **Fredrik Eriksson**, **Kristin Gustafsson**, **Pascal Dammeyer**, **Simona Vertuani** and **Shahryar Kiaii.**

The Department of Medicine, especially; **Berit Lecomte**, **Edgardo Faune**, **Elenor Nyman**, **Klas Karlsson**, **Anna Maria Berstein** and **Margaretha Wedlund** for assisting with everyday LADOK, computer and financing issues.

Apart from the above people directly involved with the completion of this thesis, there have been various friends from the scientific, entrepreneurial, academic and school fields, that have kept me going ahead during the last years, by direct supporting me, spending some quality time with me, guiding me and in short indicating how to become a better person. I would like to thank you all for being around and making my life more interesting. Below are my separate thanks to all of you.

Dr **Dimitris Dalianis** for his warm hospitality during my first winter months in Stockholm before my PhD registration and for conveying his amazing energy, Dr **Hercules Dalianis** for assisting me with all practicalities associated with my stay in Stockholm and for amazing skiing sessions in Sweden and France, **Kerstin Johansson** for being interested in my adaptation to the Swedish ‘traditions’ and the late Dr **Mando Dalianis** for promoting the idea of studying at Karolinska Institute from the early days . Many thanks to **family Dalianis**, as a whole, for allowing me to use their wonderful ‘stugor’ in the outskirts of Stockholm where manuscripts and parts of this thesis were written.

All staff and associates at *Stockholm School of Entrepreneurship (SSES)*, especially; **Rasmus Rahm** for always being there with a smile to solve all my inquiries, **Nick Kaye** for creating such a stimulating atmosphere as an SSES managing director, **Terrence Brown** for powerful mentorship, **Alf Rehn** for being the most alternative and joyful lecturer I ever had, **Lena Ramfelt** and **Tom Kosnik** for organising an excellent ‘Global Entrepreneuship Leadership’ course from the other side of the world at Stanford University, **Peter Kelly** for always having a critical approach about business financing, **Eva Runvald** for persuading me to attend the SSES Boot Camp and **Ulf Eriksson** for being such a great coach and always willing to use his network to promote your idea.

All friends and colleagues associated with *SSES* for stimulating discussions and positive criticism. Especially the Up2d8-Science team –Dr **Niklas Holmström** and **Dave Nicol**- for spending long hours on business plan creation and competitions, for some great arguments and for interesting chats over dinner, **Bhavik Gandhi** for insisting that 'you should always contact the CEO, straight away' and how to engage him into a conversation (best of luck to your ocean rowing), **Andreas Jakobsson** and **Oscar Roth** for all assistance and support during the Venture Cup competitions. **Fredrik Mannerheim**, **Adam Wern** and **Rok Berlic** for nice time in class and mingles. The Novas team of the Global Entrepreneurship Leadership course from SSES and Stanford; **Adam Jouda**, **Ali Turab Rizvi**, **Anna Swane**, **Kasey Chen**, **Manas Gupta**, **Nguyen Ha**, **Raine Lai**, **Saad Riaz** and **Sam Rahbar** for bringing in their own multinational experiences and background to every week's project. The SSES Innovation team, **Fransesca Agostino**, **Niklas Rosencrantz**, **Tomas Pangaro**, **Axel Glaessgen** for their input in designing the 'ultimate' ergonomical computer chair.

The basis of my education was formed during my time at *King's College London, University of London,* from were I graduated with a BSc in Molecular Biology and an MSc in Biomedical Science Research. I would like to thank all the staff associated with the above degrees, especially;
Dr **Mark Sanderson**, my first tutor for really believing in me from the first moment even before my official enrolment and for his continuous interest in my academic progress, Professor **Ian Sanderson** for accepting me in his laboratory and finding always time to supervise me on my BSc project, Dr **Jim Wilson** for spending long hours in the lab next to me, assisting me with everything needed for my MSc thesis, Dr **Paul Brown** for his inspiring classes and for accepting the invitation as my BSc opponent, Professor **Farzin Farzaneh** for an exciting journey in the 'Biology of Cancer' and for discussions during conferences in London and Greece, Dr **Yoshi Ohtsuka** and Dr **Nick White** for assisting me with molecular work during my BSc project, Dr **Demetra Stamm** for constantly being on my side during my project time at St. Barts Hospital, Drs **Shanta Persaud** and **Peter Jones** for organising an excellent masters degree and for always being accessible and supportive.

My experience in *London* though, would be half as rewarding, if the following were not part of my life there; Dr **Tasos Siskoglou** for introducing me on how things work in England, for hours of parallel exam studying and for a lot of computer tricks, Dr **Apostolis Siscoglou** for endless evening chats, support and fun time, the **Siskoglou family** for offering me a second place to stay, when my accommodation was not arranged, **Alexis Hatziioannou** for being an excellent caring flatmate and fun holiday time (soon we will sail race together), **Panos Ilikrinis** for an exclusive London by nights experience, **Kostas Zeikos** for sharing my passion for music and cinema, **Apostolos Mandrakoukas** for being my longest friend from the London gang, Dr **Mara Simopoulou** for

being a powerful supportive mentor, Dr **Antonis Danikas** for sharing his experience as the 'oldest' Greek student around, Dr **Giannis Serafeimidis** for his excellent biochemistry help, **Kostas Dimitrelis** for his joyful craziness, **Rigas Soldatos**, **Violetta Silva-Vargas**, **Eleni Vasilakopoulou**, **Kriton Rountos**, **Tina Lomverdou**, **Christos Tziortzios**, **Space Giannopoulou**, **Deykos Karakalpakis**, **Giorgos Mpethimoutis**, Dr **Anna Davies**, **Myrto Ranga**, **Katerina Mortoglou**, **Antonis Geralis** and **Giorgos Iliopoulos** for fun time during those years.

During the last four years of stay in *Stockholm*, I had the chance to meet some wonderful people, who have help me in their own way in completing this work; especially,

Katarina Fagerström, my best friend in Sweden, for being there from the very beginning, even before my PhD registration, believing in and supporting me till the end of this effort. Honestly, I do not have enough words to express my appreciation for everything that you have done during the last $4^{1/2}$ years. Thank you for all the amazing and memorable moments both in Stockholm and in all the places we have been to.

Jägargatan 20, is most probably one of the most known addresses for international students in Stockholm. This place has been one of the first places that I stayed in and I would like to thank the following for making it such a pleasing, multi-cultural and international environment; **Anna-Maria Psarra, Xiang Hua**, **Ia Ladestam**, **Xidan Li**, **Tara Malekshahi**, **Rafaele Attia**, **Anja Schue**, **Varinia Soledad Gaete Serrano**, **Elina Ylimaki**, **Karina Iliescu**, **Magda Leczano**, **Alexander Bondar** and **Hanna Karlsson.**

Moreover, **Alexandros Kaisar** for introducing me to an amazing group of people in Stockholm, **Eyaggelos Chandanos**, for endless academic, political and business discussions and always happy to share some Greek delicacies, **Andreas Kettis** for Flotssbro after-work skiing, barbeques, Café Opera and teaching me diplomacy, **Makis Tzortzatos** and **Aggelos Sioutas** for long coffee conversations, **Apostolis and Klairi Tsolakis** for driving 2 hours almost every Sunday in order to enjoy a cup of coffee, **Dimitra Grivogianni** for some old-time classic dinners, **Giorgos Kaponidis** for sharing his long Swedish experience, **Xristos Saripanidis** for endless political and social comparisons between Sweden and Greece, **Love Dervinger and Gun Rosvall** for renting me their flats in central Stockholm, **Josephine Wikstrand**, **Ingrid Nordberg** and **Maria Gustafsson** for memorable midsummers in the Åland archipelago, **Andrea Vlastos** for a great time during the Peloponesse summer school, **Athina Bouka** for helping me set-up one of the most successful surprise parties ever, **Vasilis Syrgiamiotis** for bringing a Greek aroma in Stockholm during the first difficult years.

During the summer time, I had the honor to work in various laboratories in my hometown Athens, in order to get accustomed to 'real' scientific world. This was an extremely valuable experience for myself as an undergraduate student at that time. For this opportunity I would like to thank; Dr **Stephen Davies**, Dr **Faii Romiou,** Dr **Vicky Sofianopoulou,** Professor **Ariadni Mavrou,** Professor **Spyros Michalas,** Dr **Efthimia Kitraki** and Dr **Antonios Andriotis.**

My old schoolmates from *Greece*, with whom I have spent some fabulous time, traveling, partying, socializing, discussing and solving everyday issues. Especially, **George Polyxronopoulos** for being so supportive especially in the beginning of my journey (good luck to your journey in Tel Aviv), **Panagiotis Koureleas** one of my first school friends for being still around when needed, **Giannis Konstantoulakis** for nice summer moments and being always so helpful, **Theofano Sarakinou** for being in the other end of the telephone line (even at 2.00AM) during all these years, **Athina Arvanitaki** for an amazing time in the States, Paris and wild summer holidays, **Hlias Pitsavos** for finally finding a common interest in entrepreneurship, **Elina Kapsimali** for great fun moments in Oxford, London and at 3.00AM waiting for the bus at Notting Hill (those were the days!), **Maria Kotaki** for always being welcome in her London flat, **Eleftheria Kallitsa** for always being supportive and interested in my progress, **Elina Dragoti** for setting an example on how to adapt and start a biology related career in Greece, **Zaira Kotaki** for her darling personality, **George Palaiokrassas** for nice leisure time, **Maira Palaiokrassa** for the good old days, **Dimitris Oikonomou** for some great party arrangements, **Aris Kossoras** for fun time during the first university years in London, **Dimitris Rakopoulos**, **Spyros Manolidis**, **Georgia Xristopoulou**, **Giorgos Papanastasiou** for nice fun times.

Furthermore, I would like to thank the following friends of mine in *Greece*; **Eyaggelos Tzoulis** for exceptional mentoring, for teaching me the importance of scheduling and of the well organised experimental thought, the late **Katerina Koroni** for treating me like a child of her own, **Xristos Prevezas**, for sharing my passion for music, for helping with party DJing (soon professionally) and for some amazing holidays, **Vasileios Prevezas** for 'not coming to study in Stockholm', **Simos Anagnostopoulos** for recommending me as an excellent candidate at King's College London and being so interested in my academic progress, **Fofo Konstantopoulou** for being such a dear cousin and finding always a way to entertain me (many thanks to all her family for being a reason to visit my mother's hometown), **Isidoros Oikonomopoulos** for teaching the art of progressive and reasoning thought in project writing, **Thanasis Karatzas** for teaching me the basis of biology, **Gely Antoniadou** for giving the 'Good Luck' teddy bear on my way to London for my first university year (nine years later, it still stands on the side of my desk), **Helen Grigoriadou** for your always thoughtfully prepared meals, **Maria Giatra**, **Karolos Frances, Maria Mpoziki**, **Ioanna Thoma**, **Alkis Argyriou**, **Katerina Mpakagianni**, **Tasos Kaitatzis**, **Vassilis Pasxonidis**, **Filaretos**

Siakos, Elena Kelegian, Kyriaki Paltatzidou and **Sergios Soursos** for some wonderful holiday moments. Dr **Michail Papamichail**, Dr **Sonia Perez**, Dr **Niki Kakoullou**, Dr **Peggy Sotiropoulou**, Dr **Maria Salagianni**, **Giannis Xondrogiannis**, Dr **Vasilis Triantis**, Dr **Rania Tsitsilonis** and Dr **Katerina Vourvouhaki** for an amazing time at the Ionian Village in South Peloponnese during two international summer schools in immunotherapy and immunology.

Finally, most importantly my *family*; my father **Vasileios** for being a role model for myself, for his extremely long hours of work in order to be able to offer me the best possible education and upbringing, my mother **Magda** for always being so caring, for constantly worrying for my well-being during my studies abroad and being so attached to me from day 1, my aunt and godmother **Theoni** for sculpting my educational background and ensuring that I have full control of my school studies, my uncle **Nikos** for his long philosophical discussions and my first -and only- cousin, **Michael**, for spending some great holiday time together from the top of the mountain to the seaside and for bringing the energy and the careless of a teenager to my life.

This thesis was supported by grants from the Swedish Cancer Society, the Swedish Foundation for Strategic Research, the Swedish Research Council and Karolinska Institutet.

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12. PAPERS I-IV