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MECHANISMS OF IMMUNE ESCAPE

Implications for Immunotherapy against cancer

Stockholm 2003

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Published and printed by Karolinska University Press
Box 200, SE-17177 Stockholm, Sweden

ISBN 91-7349-436-4

To JAM

"So let´s go where we´re happy and I meet you at the cemetry gates.....on your side"

The Smiths

Abstract

Tumor cells can be recognized and killed by cytotoxic T cells specific for certain tumor antigens. Immune mediated selection pressure along with genetic instability of tumor cells result in a growth advantage of tumor cells that acquire a less immunogenic phenotype. Increased understanding of immune escape mechanisms is crucial for the development of effective immunotherapy against cancer. This thesis deals with several aspects of immune escape and tumor induced immune suppression.

We demonstrate the paradoxical finding that IFN-γ treatment of short-term ovarian cancer cell lines (OVAC) protected these from cytotoxic T Lymphocyte (CTL) lysis. This was dependent on enhanced signaling to inhibitory NK receptors (iNKR), CD94/NKG2A, which were expressed by the CTLs. The ligand for CD94/NKG2A is HLA-E, which is a non-classical HLA class I molecule. HLA-E expression depends on its association with leader sequence peptides derived from classical and non-classical HLA class I molecules. Furthermore, the signaling capacity of the HLA-E / peptide complex is influenced by specific properties of the peptide that is bound to the HLA-E molecule. The leader sequence peptide of another non-classical HLA class I molecule, HLA-G, provides a particularly strong inhibitory signal to CD94/NKG2A receptors when bound in the peptide groove of the HLA-E molecule. Ovarian carcinomas were found to frequently express HLA-G at the protein level and HLA-E on a transcriptional level. The expression of both these molecules was induced by IFN-γ. We speculated that the increased intracellular accessibility to HLA-G leader sequence peptides (Gsp), followed by increased surface expression of HLA-E / Gsp complexes, was the underlying mechanism behind the IFN-γ mediated protection of tumor cells. In support of this possibility we were able to mimic the effect of IFN-γ by exogenously adding synthetic HLA-G leader sequence peptides to untreated tumor cells. It is concluded that IFN-γ may shift the balance towards inhibitory signaling to the CTLs, turning off the lysis of otherwise sensitive targets. The role of such ligand – iNKR interactions deserves further attention in future attempts of immunotherapy against ovarian carcinoma.

The importance of inhibitory NK receptors was further emphasized by the phenotypic analysis of ovarian tumor associated T and NK cells. There was a bias towards expression of inhibitory CD94/NKG2A receptors on the tumor infiltrating $CD56^{+}$ T cells as compared to $CD56^{+}$ T cells derived from PBL of patients and healthy donors. Further, there was an over-representation of regulatory, non-cytotoxic, $CD56^{bright}$ NK cells among the tumor associated lymphocytes. This was associated with high expression of inhibitory CD94/NKG2A receptors on this subset of NK cells. Expression of inhibitory receptors by a large number of tumor associated lymphocytes is likely to decrease their cytotoxic activity against autologous tumors.

Hydrogen peroxide, released by tumor associated macrophages severely impairs the cytokine production and cytolytic function of T and NK cells. It was demonstrated that the Th1 cytokine production of T cells with an activated/memory ($CD45RO^{+}$) phenotype was more sensitive than naive T cells to the influence of H_2O_2. Furthermore, the reduced Th1 cytokine production was associated with a block of NF-κB activation. The majority of tumor infiltrating lymphocytes is of a $CD45RO^{+}$ phenotype and our results may explain why T cells that reside in tumor lesions often display anergic properties. Based on these results we speculated that exogenous supply of antioxidants may protect immune cells from the attack of free radicals. This hypothesis was tested on 12 patients with advanced colorectal cancer who were given high doses of dietary vitamin E during a period of two weeks. In a majority of patients the supplementation of vitamin E lead to enhanced IL-2 and IFN-γ production by their T cells. Moreover, CD4/CD8 ratios were increased after treatment with vitamin E. It is concluded that the administration of dietary vitamin E may counteract tumor induced immune suppression and form an effective supplement to more specific immunotherapy.

Contents

List of original papers

This thesis is based on the following papers, which will be referred to by their roman numerals:

I. **Karl-Johan Malmberg**, Victor Levitsky, Håkan Norell, Cristina Teixeira de Matos, Mattias Carlsten, Kjell Schedvins, Rabbani Hodjattallah, Alessandro Moretta, Kalle Söderström, Jelena Levitskaya, and Rolf Kiessling. IFN-γ protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism.
Journal of Clinical Investigation 110:1515-1523 (2002)

II. **Karl-Johan Malmberg**, Håkan Norell, Kjell Schedvins, and Rolf Kiessling. Bias for expression of the inhibitory heterodimer of CD94/NKG2 among tumor associated $CD56^{+}$ T cells and $CD56^{bright}$ NK cells.
Manuscript

III. **Karl-Johan Malmberg**, Velmurugesan Arulampalam, Fumiko Ichihara, Max Petersson, Kazutaki Seki, Tove Andersson, Rodica Lenkei, Giuseppe Masucci, Sven Pettersson, and Rolf Kiessling. Inhibition of activated/memory ($CD45RO^{+}$) T cells by oxidative stress associated with block of NF-κB.
Journal of Immunology 167: 2595-2601 (2001)

IV. **Karl-Johan Malmberg**, Rodica Lenkei, Max Petersson, Tomas Ohlum, Fumiko Ichihara, Bengt Glimelius, Jan-Erik Frödin, Giuseppe Masucci, and Rolf Kiessling. A short-term dietary supplementation of high doses of vitamin E increases T helper 1 cytokine production in patients with advanced colorectal cancer.
Clinical Cancer Research 8: 1772-1778, June (2002)

Abbreviations

APC	Antigen presenting cell
β_2m	B_2-microglobulin
B7sp	HLA-B7 signal sequence derived peptide (VMAPRTVLL)
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain protein
FITC	Fluoreoscein isothiocyanate (fluorochrome)
FLIP	FLICE inhibitory protein
HA-2	Histocompatibility-2
HLA	Human leukocyte antigen
IFN	Interferon
ILT	Immunoglobulin-like transcript
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KARAP	Killer cell–activating receptor–associated polypeptide
KIR	Killer immunoglobulin-like receptor
LAK	Lymphokine activated killer
LIR	Leukocyte immunoglobulin-like receptor
LOH	Loss of heterozygosity
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MICA/B	MHC class I-related chain A/B
NCR	Natural cytotoxicity receptor
NF-κB	Nuclear Factor-κB
NK	Natural killer
OVAC	Short-term ovarian carcinoma cell line
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cells
PTP	Protein Tyrosine Phosphatase
R-PE	R-phycoerythrin (fluorochrome)
RT-1	Rat-1
TAA	Tumor associated antigen
TAL	Tumor associated lymphocyte
TAP	Transporter associated with antigen presentation
TCR	T cell receptor
TGF	Tumor growth factor
Th	T helper
TIL	Tumor infiltrating lymphocyte
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
ULBP	UL16 binding protein

PREFACE

This thesis deals with mechanisms of immune escape by tumors. The appearance of such immune escape mechanisms implies that the immune system participates in the selection pressure that "shapes" the phenotype of tumor cells and suggests that there is some sort of tumor immune surveillance. Around two years ago when I presented some of my work to Georg Klein during one of the regular meetings between Rolf and Georg (The Georg meeting) he taught me an important lesson. Tumors are not intelligent. Escaping from host immunity is not, as it may sound, an active process of smartness by the tumor. Citing from Georg's summary of a conference on spontaneous regression of cancer held at The Johns Hopkins Medical Institutions in 1974 (7): "Selection favors the phenotype, i.e., the cell that can grow in spite of the immune rejection process". Hence, selection is not the underlying mechanism. Bearing this in mind it is my intention to review the evidence for such an immune selection pressure and describe some of the mechanisms that may allow tumors to grow despite a functional immune system and even after active immunisation. Thereafter I will discuss the results presented in the four included papers with a general attempt to outline possible implications for immunotherapy against cancer.

The work included in this thesis is the result of four years of work in the laboratory of Rolf Kiessling. It reflects our group's broad interest in tumor immunology spanning from detailed mechanistic aspects of immune regulation to the general attempt to bring immunotherapy to the clinic. I find it hard to define a limited number of specific aims that my work has been based on. Throughout the years we have initiated many projects based on experimental discoveries presented to us from the scientific literature or from our own incidental findings in the daily laboratory work. Most of these efforts remain unpublished but have probably contributed just as much to my scientific development.

Apart from being proud to be a member of a research group and have my picture posted on the internet, I do not remember having any particular expectations when joining the group in 1998. I do not know if my contribution to science will be of any "long-lasting" significance. Time will tell the *"in vitro"* artifacts from the "*real*" findings. However, I do know the impact of science on my life. Wherever my future position will be, in the clinic or in a laboratory, I hope to preserve the feeling of humbleness to the complexity of biological knowledge and to the short-lived nature of truth.

Bromsten, 24/12 2002

INTRODUCTION

The Immune system

The ability of humans to survive infections of pathogens is the result of a functioning immune system. It is not my intention to cover the complex biology of all components and strategies used by the immune system to fulfill this task. Here, I will describe some of the basic components and concepts of immunity of particular importance for the work described in this thesis. These include: i) MHC molecules ii) antigen processing and presentation and iii) NK receptors and their ligands.

The major histocompatibility complex

The major histocompatibility complex (MHC) was first described in mice as the major locus determining the outcome of tissue transplants (8). The MHC antigens were discovered on leukocytes and are therefore in humans referred to as human leukocyte antigen (HLA). Equivalents in mice and rats are termed histocompatibility-2 (H-2) and Rat-1 (RT-1) respectively (9). The importance of this gene complex to adaptive immunity was clearly demonstrated by Rolf Zinkernagel and Peter Doherty in their description of the MHC restriction phenomenon (10). They showed that virus specific T cells recognized viral antigens together with self MHC proteins. Later, others have demonstrated that the antigens are processed into smaller fragments, peptides, which are bound to the MHC molecules (11-13). TCR mediated recognition of foreign peptides in the context of MHC is required for T cell activation and forms the basis for the adaptive immune response. $CD8^+$ T cells interact with MHC class I molecules presenting peptides derived from self-proteins or intra cellular pathogens, while $CD4^+$ T cells recognize peptides presented on MHC class II molecules. MHC class I molecules are expressed by most nucleated cells in the body (14). In contrast, MHC class II molecules are selectively expressed on APC, such as DC, macrophages and B cells (15).

MHC class I and class II genes are polygenic (several gene loci) and polymorphic (multiple alleles). MHC alleles of each gene loci are co-dominantly expressed so that each individual can express several different MHC class I and class II proteins. In humans there are three HLA class I loci (HLA-A, -B and –C) and three HLA class II loci (HLA-DP, -DQ and –DR). In addition, the MHC gene loci include "non-classical" MHC class I molecules (MHC class Ib molecules). In humans, the encoded proteins of these loci include HLA-G, HLA-E and HLA-F. HLA-G and HLA-E function as ligands for receptors that regulate the cytotoxic activity of T and NK cells as will be detailed later in this thesis (16,17). However, non-classical MHC class I molecules may also be involved in presentation of antigens, although knowledge of such functions remains limited. A potential role for HLA-E as a restriction element for T cells with anti-mycobacterial activity was recently demonstrated (18).

When the crystal structure of the MHC class I molecule was resolved it clarified several important biological features of T cell recognition of MHC class I complexes (19,20). The MHC class I / peptide complex consists of a heavy chain that is non-covalently linked to a β_2-microglobulin (β_2m) subunit and a peptide of 8-11 amino acids (aa) (**figure 1**). The α1 and α2 domains of the heavy chain form the sides of the

peptide binding groove and the α3 domain has an immunoglobulin like fold and anchors the complex at the cell membrane. The β_2m subunit interacts with all three domains of the heavy chain and is crucial for the stability of the MHC class I complex (21). The high number of specificities recognized by the TCR of $CD8^+$ T cells is a combination of structural features of the peptide binding groove and the aminoacid composition of the peptide (22).

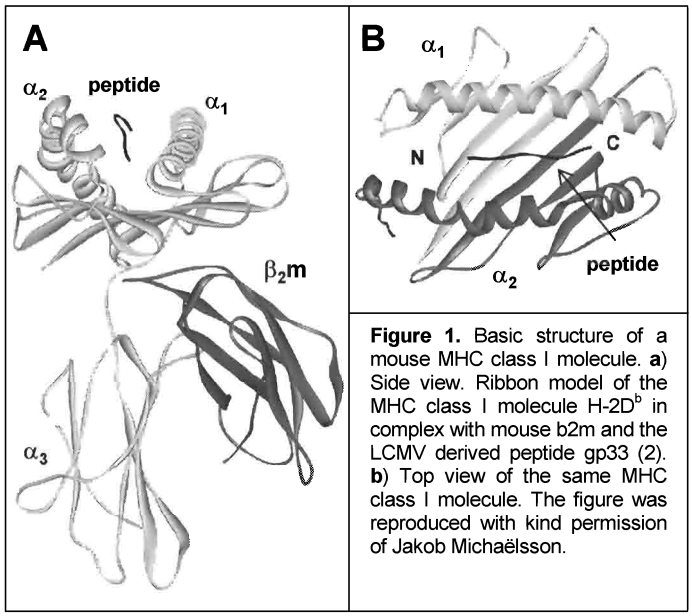

Figure 1. Basic structure of a mouse MHC class I molecule. **a**) Side view. Ribbon model of the MHC class I molecule H-$2D^b$ in complex with mouse b2m and the LCMV derived peptide gp33 (2). **b**) Top view of the same MHC class I molecule. The figure was reproduced with kind permission of Jakob Michaëlsson.

Antigen processing and presentation

The machinery responsible for generating peptide/MHC complexes at the cell surface involves a large number of complex biochemical events and has become a field of research in itself. All the molecules involved in antigen presentation are of fundamental importance for the generation of any type of immunity. Here, I will outline some of the main characteristics of the MHC class I and II antigen processing and presentation pathways.

Antigen processing and presentation involves six fundamental steps that differ for MHC class I and class II restricted pathways of antigen presentation (**figure 2** and refs. 23,24). These include: i) acquisition of antigen, ii) tagging of antigen for degradation, iii) proteolysis, iv) delivery of peptide to the MHC molecule, v) binding of peptide to the MHC molecule and vi) display of MHC complexes at the cell surface. The key distinction between MHC class I and MHC class II restricted pathways is the topological compartment sampled by either pathway. MHC class I molecules present peptides derived from intracellular proteins of self or pathogenic origin (although

exceptions to this rule occurs, that is presentation of exogenous antigens on MHC class I molecules, refs. 25,26). Ubiquitin-tagged proteins are degraded into peptides by the barrel-shaped catalytic core of the 26S proteasome (27). The catalytic activity resides in the two inner β-rings that are surrounded by two stabilizing α-rings. The peptides are then translocated into the endoplasmic reticulum (ER), via transporters associated with antigen presentation (TAP), where they are assembled with the MHC class I molecules with the assistance of a chaperon, tapasin (28). The fully assembled MHC complex now leaves the ER and travels straight to the cell surface.

MHC class II expressing APCs acquire extra cellular proteins by phagocytosis, pinocytosis or endocytosis (29). Proteins are prepared for degradation by means of physicochemical reduction of disulfide bonds in acidic endocytic vesicles (30). Generation of peptides occurs by the enzymatic activity of a large number of cathepsins that are present in the lysosomes (31). MHC class II molecules are guided to the lysosome by a chaperon, the invariant chain, Ii (32). The invariant chain is degraded by proteolysis leaving a remnant, the CLIP peptide that is exchanged with the antigenic peptide by the catalytic aid of HLA-DM. The peptides binding MHC class II molecules are longer than those presented on MHC class I molecules and protrude outside the peptide binding groove.

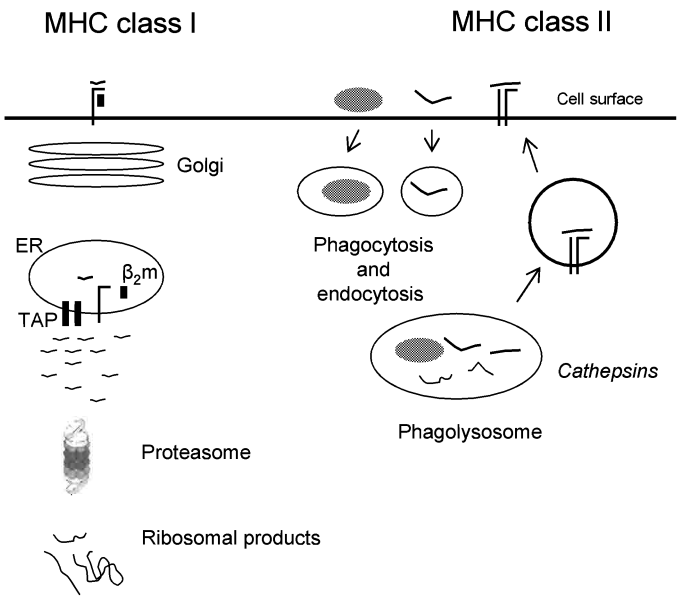

Figure 2. Schematic overview of the MHC class I and class II antigen processing and presentation pathways. Adapted from Grommé et. al. (4) and Kessler et. al.(6)

MHC class I antigen processing and presentation can be affected by pro-inflammatory cytokines. Both IFN-γ and TNF-α induces coordinated changes in several components of the antigen presentation pathway leading to enhanced expression and stability of MHC class I / peptide complexes at the cell surface (23,33). One molecular event that explains this is the cytokine induced exchange of three subunits of the proteolytic core of the proteasome β1, β2 and β5 into LMP2, MECL1 and LMP7 (34,35). This leads to a different cleavage specificity of the proteasome, which results in peptides that bind more efficiently to the MHC class I molecule (36,37). Also the expression of MHC class I, TAP and Tapasin is increased by proinflammatory cytokines, facilitating TCR mediated recognition by $CD8^+$ T cells.

NK receptors and their ligands

When inhibitory NK receptors (iNKR) were described on mouse and human NK cells (38,39), it provided a molecular explanation for the "missing self hypothesis" postulated by K. Kärre (40,41). The capacity of NK cells to kill targets that missed "self" MHC class I molecules was due to the lack of inhibition via MHC class I binding receptors. Subsequently it has been shown that NK cells are also dependent on triggering signals provided via ligation of activating receptors (42,43). The family of receptors governing the functional activity of NK cells has grown immensely and includes a large number of receptors with different specificities (**table 1**, ref. 1).

Table 1. *NKR and their ligands*

NKR	CD/other	Signal	HLA ligand
KIR2DL1	CD158a/p58.1	Inhibitory	HLA-C group 2
KIR2DS1	CD158h/p50.1	Activating	HLA-C group 2
KIR2DL2	CD158b1/p58.2	Inhibitory	HLA-C group 1
KIR2DL3	CD158b2/p58.3	Inhibitory	HLA-C group 1
KIR2DS2	CD158j/p50.2	Activating	HLA-C group 1
KIR2DL4	CD158d/p49	Potential for both	HLA-G
KIR2DL5	CD158f	Potential for inhibition	?
KIR2DS4	CD158i/p50.3	Activating	?
KIR2DS5	CD158g	Potential for activation	?
KIR3DL1	CD158e1/p70	Inhibitory	HLA-Bw4
KIR3DS1	CD158e2/p70	Potential for activation	?
KIR3DL2	CD158k/p140	Inhibitory	HLA-A3, -A11
KIR3DL7	CD158z	Potential for inhibition	?
LIR-1	CD85j	Inhibitory	HLA-G, HLA-A and -B
CD94/NKG2A	-	Inhibitory	HLA-E
CD94/NKG2C, -E/H	-	Activating	HLA-E
NKG2D		Activating	MICA, -B, ULBP-1, -2, -3
NKp46		Activating	?
NKp44		Activating	?
NKp30		Activating	?

Activating and Inhibitory NK receptors. 2D and 3D designate KIR with two or three Ig domains respectively. L and S refer to long and short cytoplasmatic tails, respectively. Group 1 alleles include HLA- Cw1, w3, w7, w8 while group 2 include HLA-Cw2, w4, w5, w6.

Interestingly, many of these NKR are also expressed by activated and/or memory T cells (44). The role of iNKR and their ligands in tumor immunity will be discussed in detail later in this thesis with reference to **paper I** and **II**. Here, I will briefly introduce the different families of human NKR and their ligands.

Two structurally distinct classes of human HLA class I binding NKR have been identified. One class of receptors belongs to the Ig-like super family. This class includes the killer cell Ig-like receptors (KIR) and the leukocyte Ig-like receptor-1 (LIR-1), which is also known as Ig-like transcript-2 (ILT2) and CD85j in the most recent nomenclature (45). There are approximately 13 KIR family members and these are variably expressed in different donors (46). The second class comprises heterodimers formed by the C-type lectin-like molecules CD94 and NKG2A, -C or –E (17,47).

Within both groups there are inhibitory and activatory variants (**figure 3**). Inhibitory KIRs (KIRDL) have a long cytoplasmic tail that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain. When the inhibitory motifs become phosphorylated they recruit the tyrosine phosphatases SHP-1 and SHP-2 that mediates the inhibitory signal (48). In contrast, activating KIRDS have short intracytolplasmic domains and lack inhibitory motifs. Upon ligation, activating KIRs associate with an adaptor molecule termed DAP12 (also known as KARAP) (49,50). DAP12 carries immunoreceptor tyrosine-based activating motifs (ITAM) and transmits the activation signal further downstream in the receptor bearing cell. Ligation of the CD94/NKG2 heterodimer results in inhibition (NKG2A) or activation (NKG2C, E/H) depending on the covalently associated NKG2 molecule (51-53).

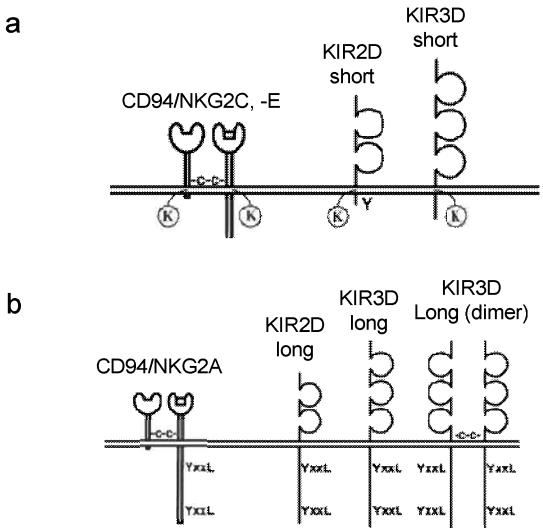

Figure 3. Activatory and Inhibitory receptors. **a**) Activatory receptors containing a short cytoplasmatic domain. **b**) Inhibitory receptors with long intracytoplasmatic domains carrying ITIMs (YxxL). Adapted from Lanier (1).

Ligands for the KIR are the classical HLA class I molecules HLA-A, -B, -C. Different KIR recognizes different groups of HLA alleles sharing structural features at the α1 domain (54-56). The site of interaction between KIR and HLA molecules overlaps partially with that of the TCR of $CD8^+$ T cells. This is illustrated in **figure 4** showing the co-crystal of HLA-Cw3 and KIR2DL2 and that of H-$2K^b$ and 2C TCR.

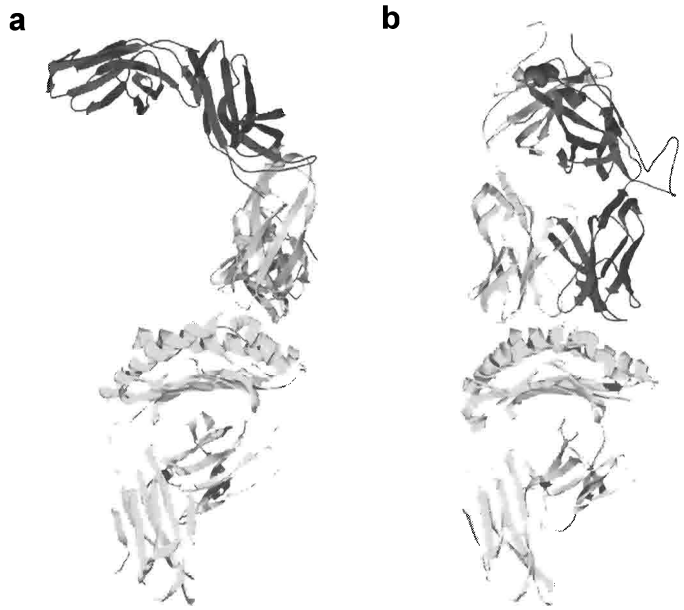

Figure 4, Crystal structures showing the molecular interactions of MHC class I molecules with KIR and TCR. **a**) HLA-Cw3 – KIR2DL2 (PBD 1EFX). **b**) T cell receptor 2C – H-$2K^b$ (mouse MHC class I)(PDB 2CKB). The figure was reproduced with kind permission of Jonas Sundbäck

LIR-1 recognizes several classical HLA class I alleles but also the non-classical HLA class I molecule HLA-G. HLA-G is physiologically expressed in the placenta and has been proposed to play a role in protecting the fetus from invading maternal NK cells (57). It is also expressed on medullary and sub capsular epithelial cells in the thymus suggesting that it may influence the selection of the T cell repertoire thereby explaining the T cell tolerance to HLA-G (58). By alternative splicing seven different isoforms of HLA-G are generated, four of which are membrane bound (HLA-G1, -G2, -G3 and –G4) and 3 soluble secreted isoforms (HLA-G5, -G6 -G7) (59). Ectopic expression of these molecules have also been reported on various types of neoplasias, possibly reflecting a way for tumors to escape recognition by $iNKR^+$ NK and T cells (see further on page 27 and refs. 60-62). The HLA-G molecule presents a broad array

of peptides although its function as restriction element for T cells remains unclear (63-65).

The major ligand for both the activatory and inhibitory isotypes of CD94/NKG2 is HLA-E in man and Qa-1 in mouse (66-68). HLA-E and Qa-1 expression depends on its binding to hydrophobic nonamer peptides derived from the leader sequences of certain HLA class I molecules as well as that of HLA-G (69-73). HLA-E binding peptides share a common motif: methionine at position 2, and leucine or isoleucine at position 9 (**table 2**, ref. 73)

Table 2. *HLA-E binding peptides*

HLA allotype	Leader peptide sequence	K_D (μM) (CD94/NKG2A)	Inhibition of NK lysis
HLA-G	VMAPRTLFL	2	Yes
HLA-Cw*0302	VMAPRTLIL	7	Yes
HLA-B*0702	VMAPRTVLL	11	Yes
HLA-B*5801	VTAPRTVLL	17	No
HLA-Cw0702	VMAPRALLL	>34	No
HLA-Cw*0402	VMEPRTELL	>75	No

Peptide residues of HLA class I leader sequences and their capacity to confer binding of HLA-E to CD94/NKG2A. Correlation with inhibitory function of NK lysis. Adapted from Valéz-Gómez et. al. (74).

It has been demonstrated that the strength of the inhibitory signal depends on properties of the leader sequence peptide that is bound to the HLA-E molecule (74). While some peptides mediate strong interaction with the CD94/NKG2 heterodimer and thereby promote significant positive or negative signaling, other peptides seem to silence this pathway (75). Protective peptides gain access to the HLA-E molecule and bind stably in its peptide binding groove due to the presence of HLA-E binding motifs in position 2 and 9. However, protection is also influenced by peptide residues that face the CD94/NKG2A receptor. It has been suggested that position 5 is particularly important for the interaction with the receptor. A change in this position from argenine to valine (R5V) in Qa-1 and HLA-E binding peptides abrogated the recognition by CD94/NKG2A (75,76). The peptide dependency and the hierarchy of leader sequence peptides are illustrated in **figure 5**. The HLA-G leader sequence derived peptide (Gsp) promotes a strong interaction with CD94/NKG2 receptors (74), which is of particular interest to our studies. The Gsp is identical in position 2, 5 and 9 to other protective peptides, such as HLA-B7sp, why its superior inhibitory capacity may depend on aminoacids in position 7 and 8 (**table 2**).

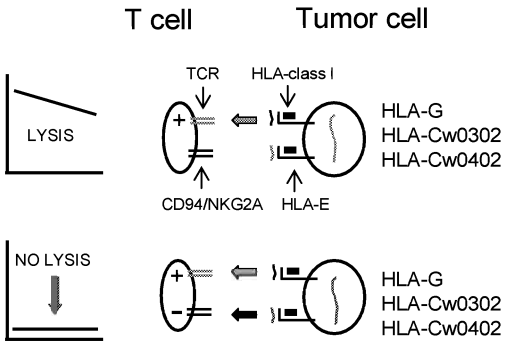

Figure 5. Peptide hierarchy of HLA-E binding peptides. Certain leader sequence peptides bind the HLA-E molecule and provide strong inhibitory signaling via CD94/NKG2A receptors turning of the lysis of target cells. The negative signal may override the positive signaling of the TCR/HLA class I interaction.

The CD94/NKG2 - HLA-E interaction represents a non-overlapping receptor-ligand system to HLA-G – LIR-1 by which cells recognize HLA-G expressing cells (70). This is illustrated in **figure 6**.

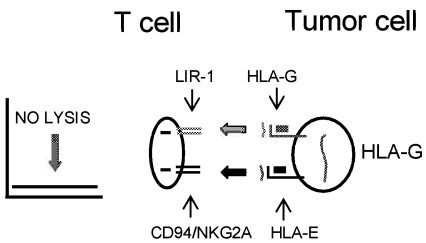

Figure 6. Depicted are the two partially overlapping receptor-ligand systems sensing the expression of HLA-G.

NKG2D is an activating receptor that is divergent from the other NKG2 receptors (77). It is widely expressed by NK cells, γδ, and $CD8^+$ T cells and recognizes stress induced ligands such as the MHC class I homologous MICA and MICB in humans and Rae-1 and H-60 in mouse (77,78). Other ligands for NKG2D in humans are the UL-16 binding protein (ULBP). NKG2D engagement triggers NK cells and co-stimulates antigen specific T cells (77,79,80). These two distinct tasks were recently shown to be mediated by association with different signaling molecules (81). While direct killing was mediated by DAP10 association, DAP12 was involved when NKG2D co-stimulated T cell functions. NKG2D mediated co-stimulation during a primary tumor challenge aids in generating long-term anti-tumor CD8 T cell responses that reject NKG2D ligand negative tumors upon a subsequent challenge (82). MICA and MICB are frequently expressed on epithelial tumors emphasizing its potentially useful role for immunotherapy against cancer (79,83-85). Another set of activating receptors with yet unknown ligands have been identified. These are the natural cytotoxicity receptors (NCR), including NKp46, NKp44 and NKp30. These receptors are selectively expressed on NK cells and have been shown to participate in the recognition of several human tumors (86-89).

Tumor immunology

Immune surveillance

In the early 20^{th} century the immunologist Paul Ehrlich first conceived of the idea that tumors arise continuously in our bodies and that the immune system scans and eradicates these tumors before they become clinically manifested (90). Experimental evidence that tumors could be repressed by the immune system came from the use of tumor transplantation models in the mid 20^{th} century. Several researchers observed that it was possible to protect mice from a lethal tumor challenge by vaccinating with lysed / killed tumor cells (91,92). Although the earliest evidence of tumor rejection of transplantable tumors could be largely explained by the allograft rejection, subsequent studies in syngenic mice showed that strong and protective immune responses could be evoked also without allogeneic barriers (91,93-95). The results from tumor transplantation models strongly suggested the existence of tumor associated antigens (TAA) and formed the basis of the 'immune surveillance theory' postulated by Sir Macfarlane Burnet and Lewis Thomas (96-98). According to this theory, spontaneously developing carcinomas are efficiently eliminated by the immune system recognizing specific determinants expressed by the tumor cells. Burnet claimed that the presence of such a tumor scanning system was an evolutionary necessity and as important for survival as the protection against infections.

Soon, critique against this concept aroused. Experimental data challenged obvious predictions of the immune surveillance theory. There was no increased incidence of spontaneous or chemically induced tumors in athymic, nude mice, as compared to wild type animals even though long observation periods of up to 420 days were employed (99-102). If tumors eventually appeared in immunocompromised mice these were often virally induced lymphomas (93). Furthermore, several transplantable tumors were non-immunogenic when transplanted into syngenic hosts, casting doubts over the existence of tumor antigens (103,104). All these lines of evidence argued against immunological surveillance.

Immunoediting-Revival of concept

Recent evidence from mice that lack components of the IFN-γ signaling pathways has revived the discussion of a potential immune surveillance against tumors (105-107). IFN-γ receptor or signal transducer and activator of transcription (STAT-1) deficient mice were shown to be 10-20 times as sensitive to methylcholanthrene (MCA) induced tumor formation when compared to wild type mice (107). STAT-1 is a transcriptor factor that mediates IFN-γ receptor signaling (108,109). In addition, mice that lacked perforin, one of the effector molecules mediating T and NK cell lysis, were more sensitive to MCA induced tumor formation (110-112). Further evidence for the involvement of lymphocytes in this "immune surveillance" came from studies in mice lacking recombination activating gene 1/2 (RAG-1/2). These mice have no functional T and B cells in contrast to the nude mice in which low frequencies of functional αβ- and γδT cells are present (113-115). RAG-2 KO mice developed MCA induced fibrosarcomas more rapidly and at a higher frequency than wild type animals (105). Interestingly, 26 out of 26 RAG-2 deficient mice also developed spontaneous epithelial tumors of predominantly intestinal origin. The susceptibility to tumor formation in various strains of immunodeficient mice is shown in **table 3**.

Similar to the findings in early mouse models of immune deficiency the immune surveillance theory was criticized due to the lack of enhanced tumor incidence in immune compromised humans. Evidence from long-term follow-up studies of immunosuppressed transplant and AIDS patients showed that they developed virally induced tumors at an unusually high rate, but this was not in support of the existence of immune surveillance against tumors (117-119). However, also the risk of developing non-virally induced tumors such as melanomas, lung, colon, bladder, kidney and endocrine tumors has been reported to be higher in transplanted patients (119,120). Further support of immune surveillance against tumors in humans is provided by the positive correlation between the presence of lymphocytes in a tumor as detected by immune histochemistry, and increased patient survival (121-124).

The experimental revival of the immune surveillance concept has extended the phenomenon and is now referred to as: cancer immunoediting (105,116). Cancer immunoediting includes both immune surveillance and immune escape. It refers to the protective capacity of the immune system against tumors (i.e. immune surveillance) and to the consequences of the proposed immune selection pressure (i.e. immune escape). The concept of cancer immunoediting brings little novelty to the understanding of immune recognition of tumors apart from giving a somewhat wider definition of immune escape, including the observation that tumors that grow in immunocompromised hosts become more immunogenic and are easily rejected when transplanted into immunocompetent mice (105,125,126). In the absence of immune selection, tumors may develop an immunogenic phenotype.

Table 3. *Enhanced susceptibility of immunodeficient mice to formation of chemically induced and spontaneous tumors*

Phenotype or depletion	Immunodeficiency	Tumor susceptibility
RAG[1]-2$^{-/-}$	T, B and NKT[2] cells	MCA[3]-induced sarcomas Spontaneous intestinal neoplasia
RAG-2$^{-/-}$ * STAT 1$^{-/-}$	T, B and NKT cells, insensitive to IFN-γ and IFN-α/β	MCA-induced sarcomas, Spontaneous intestinal neoplasia
BALB/c SCID[4]	T, B and NKT cells	MCA-induced sarcomas
Perforin$^{-/-}$	Lack of Perforin	MCA-induced sarcomas Spontaneous disseminated lymphomas
TCR Jα281$^{-/-}$[2]		
Anti-NK1.1 antibody	NK and NKT cells	MCA-induced sarcomas
Anti-Thy1[5] antibody	T cells	MCA-induced sarcomas
αβ T cell$^{-/-}$	αβ T cells	MCA-induced sarcomas
γδ T cell$^{-/-}$	γδ T cells	MCA-induced sarcomas DMBA[6]/TPA[6]-induced skin tumors
STAT1$^{-/-}$	Insensitive to IFN-γ and IFN-α/β	MCA-induced sarcomas
IFN-γ-receptor1$^{-/-}$	Insensitive to IFN-γ	MCA-induced sarcomas
IFN-γ$^{-/-}$	Lack of IFN-γ	MCA-induced sarcomas C57BL/6[7]: Spontaneous disseminated lymphomas BALB/c[7]: Spontaneous lung adenocarcinoma
Perforin$^{-/-}$ * IFN-γ$^{-/-}$	Lack of perforin and IFN-γ	MCA induced sarcomas Spontaneous disseminated lymphomas
IL12$^{-/-}$	Lack of IL-12	MCA-induced sarcomas

[1] RAG (recombination-activating gene), [2] NKT cells are defined by their restriction to CD1 and limited TCR Vα.chain usage: Vα14-Jα281 in mice and Vα24 in humans, [3] MCA (methylcholanthrene), [4] SCID (severe combined Immunodeficiency), [5] Thy1 (antigen expressed by mouse T cells), [6] DMBA (7,12-dimethylbenzanthracene), TPA (12-*O*-tetradecanoylphorbol-13-acetate), [7] Two commonly used strains of mice.
The table was adopted from Dunn et.al. (116).

Evidence for immune escape upon immune mediated selection pressure is abundant both in mice and humans. Re-passage of transplantable tumors through immunocompetent hosts leads to the development of tumor variants with reduced immunogenicity (127,128). In humans, the immune selection pressure combined with the genetic instability of tumor cells frequently give rise to deletions and/or altered expression of several molecules important for immune recognition, such as components of the antigen processing and presentation pathway (129,130). Immune escape mechanisms and the development of escape variants is the focus of this thesis and will be discussed in detail in a separate chapter.

Several aspects of the original version of the immune surveillance theory were probably incorrect and most tumor immunologists would agree that it is not a primary property of the immune system to fight cancer. From an evolutionary perspective there is no advantage of protecting a species from a disease occurring after fertile age (131).

Most tumors arise late in life when the evolutionary pressure can not exert its function. Nevertheless, as outlined above, most tumors are antigenic and can be eradicated by the immune system given the right circumstances (132). Lack of immunogenicity is therefore a result of the tumor's inability to induce an immune response. Understanding the particular conditions under which anti-tumor immune responses can be raised is the main challenge for cancer immunotherapy.

Immunotherapy against cancer

Immunotherapy against cancer is applied tumor immunology, probing the efficacy of new concepts of cellular and molecular immunology. Steven Rosenberg, one of the leading researchers in this field, entitled a recent review on tumor immunology and immunotherapy as follows: *"A new era for cancer immunotherapy based on genes that encode cancer antigens"* (133). The "new era" refers to breakthroughs in understanding of the molecular mechanisms responsible for immune recognition and the advent of recombinant DNA technologies. Although anecdotes tell that immunization attempts against cancer were performed during the 18th century, it is not until the last decade that the antigens and the T cell receptors responsible for immune recognition of tumors have been identified. Still, the progress of vaccination against cancer is dependent on new insights in the fields of basic cellular and molecular immunology. Fundamental properties of the immune system such as formation of long-term memory, tolerance, immunodominance and anergy remain to be fully understood. The use of mature dendritic cells both to break tolerance when trying to induce tumor immunity (134) and to induce peripheral tolerance in the case of autoimmune disorders (135) clearly demonstrates that more knowledge is needed to elucidate basic mechanisms of immune activation.

Immunotherapy is usually divided into non-specific versus specific, and passive versus active, depending on the strategy used. An example of clinically used non-specific and passive immunotherapy is the application of Bacillus Calmette-Guerin (BCG) in patients with urinary bladder carcinoma (136). Other non-specific, passive strategies involve administration of immunostimulatory cytokines such as interleukin-2 (IL-2) and IFN-γ. IL-2 therapy alone can lead to complete and partial regression of large invasive tumors but only in a limited number of patients (137,138). Based on the discovery and identification of tumor associated antigens, hope is to target tumors specifically by antibodies or by CTLs. Here I will focus on the development of T cell based therapies. These include specific vaccination and adoptive transfer of "*in vitro*" generated anti-tumor CTLs. Vaccination may be based on tumor antigens in the form of peptides, protein, naked DNA or mRNA. In DC based vaccines these antigens are pulsed or transduced into dendritic cells that are adoptively transferred into patients. DC based cancer vaccines were thoroughly described in a doctoral thesis by A. Lundquist defended January 2003 at the Karolinska Institutet and is beyond the scope of this dissertation (139). Pilot studies and phase I clinical trials have tested several of these strategies with a varying degree of success (134,140-142). It is obvious that much improvement is needed before immunotherapy will be a major force in a clinical setting. The salient issues that require resolution for improving the feasibility, clinical efficacy and outcome of T cell based therapies are discussed below.

Which antigen(s) should be targeted?

A "tailored" tumor antigen should ideally have the following characteristics: It should be highly tumor specific so that an immune response runs little risk of generating autoimmunity. It should contain immunodominant epitopes accessible for both MHC class I and II presentation pathways, eliciting strong immune responses and formation of long-term memory. It should be expressed by several tumor types enabling the generation of a common anti-cancer vaccine. It should be expressed by all tumor cells in a tumor lesion and its expression should be essential for tumor cell growth.

During the last decade a large number of more or less tumor specific antigens have been defined (133,143). Although none fulfill all of the criteria presented above, some have considerable potential for generating anti-tumor immune responses. The tumor associated antigens can be divided into different groups depending on their specific characteristics. They come from normal, non-mutated genes whose expression is limited to tumors and a few selected normal tissues. They can be products of mutations related to the oncogenic process or of viral origin. Several different strategies have been used to discover new potential tumor antigens (133). Most strategies rely on elucidating the recognition pattern of anti-tumor CTLs, or anti-tumor antibodies derived from cancer patients (i.e., SEREX). Biochemical approaches, such as eluting peptides from HLA molecules, and strategies involving "*in vitro*" sensitization of T cells with candidate antigens (i.e., reverse immunology approach) have also contributed to the increasing list of potential tumor antigens (144-146). The details of these strategies are beyond the scope of this thesis and will not be described here.

The choice of a particular antigen for a clinical vaccination trial would depend on its ability to fulfill the list of criteria presented above. Truly tumor specific antigens have a very restricted expression pattern (for some even patient specific). We are facing a modified version of the "uncertainty principle", originally defined by the physicist Werner Heisenberg: 'What you gain in specificity will be lost in generality'. When reviewing the spectra of tumor antigens Eli Gilboa ordered the antigens according to their usefulness as tumor rejection antigens (143). In principle his operational ranking was based on the likelihood that moderate to high avidity T cells are present and can be stimulated by a vaccination procedure. This would rank self antigens as poor candidates, since high avidity T cells are likely to be deleted in the thymus or anergic due to peripheral tolerogenic mechanisms. Although there is evidence that immunization can be effective even if the response is limited to low avidity CTLs (147), overcoming central and peripheral tolerance will be a key problem for the use of most tumor antigens. This was further emphasized in a multi-peptide vaccine using two peptides from the cancer-testis NY-ESO-1 antigen (148). Although high numbers of peptide specific T cells were generated they did not recognize tumor cells. These low quality (low avidity) CTLs were probably generated because of excess amounts of peptides in the vaccine formulation (149). Furthermore, one of the included peptides gave rise to responses against two cryptic epitopes, i.e. epitopes that are not naturally presented by the tumor cells (150). These responses undermined those directed against the original epitope leading to the generation of non-tumor reactive CTLs.

Attempts have been made to create more "ideal" antigens by molecular manipulation. By inserting an amino acid change in the peptide epitope it is possible to produce an antigen that binds stronger to the relevant HLA molecule and therefore stands higher chances of breaking tolerance against self proteins (140,151,152).

Vaccination of melanoma patients with a modified peptide, gp100:209-217 (210M), from the gp100 melanocyte differentiation antigen was able to induce an immune response that recognized the native peptide antigen and HLA-A2 matched tumors. In combination with IL-2 this peptide induced objective clinical responses in 13 out of 42 treated patients (140).

A strong immune response against one single epitope will most likely result in antigenic loss variants, i.e. the outgrowths of tumor cell clones that have lost the expression of the antigen targeted by the immune system. To avoid this, several researches have suggested the use of multi-epitope vaccines. Successful attempts have been made using genetic constructs coding for a series of epitopes ordered in a "string of beads" fashion (153-155). The clinical usefulness of such vaccines remains to be established. It seems though as if every solution leads to a new problem. The multi-epitope solution leads to the problem of immunodominance: Immune responses to immunodominant epitopes within a multi-epitope construct may undermine responses to subdominant epitopes. How should the multiple epitopes be presented to the immune system in order to induce strong immune responses against each individual epitope? One approach may be to target the multi-epitope construct for proteasomal degradation via ubiquitination (154). Furthermore, it has been shown that patterns of immunodominance among viral epitopes can be altered by pulsing individual peptide antigens onto DC compared to vaccinating with the peptide mixture (156). The factors governing immunodominance are largely unknown but are important to elucidate as they influence the efficacy of multi epitope vaccination against cancer.

Mimicking infection, a question of tumor localization?

Several seemingly simple questions concerning vaccination against cancer lack reliable answers. It is not known whether one should vaccinate intramuscularly, intradermally or even intralymphatically. It is also unclear which adjuvants should be included in the vaccine formulation. It has been suggested that a vaccine should mimic the situation of an infection providing a "danger signal" to the body (157). The presentation of sterile peptides in the absence of antigen presenting molecules may actually lead to T cell tolerance and enhanced tumor growth (158,159). The role of tumor localization during T cell induction was recently studied in several different tumor models (160). It was demonstrated that efficient anti-tumor immunity was dependent on sufficient tumor cells reaching the secondary lymphatic organ early and for a long time period. Tumors that did not reach the lymphatic organs or those that invaded diffusely were either ignored or they induced T cell deletion. Interestingly, expression of co-stimulatory molecules by the tumor cells did not influence the T cell priming but helped in eradicating the tumors during the effector phase of a primed immune response. In another study, intralymphatic DNA vaccination was shown to enhance the immunogenicity 100 to 1000 fold when compared to intramuscular and intradermal vaccination, indicating that this largely neglected issue may be very important to consider (161).

Immunotherapy of "late stage" disease?

To date, most vaccinations against cancer have been performed in the late stages of the disease when all other treatment modalities have failed. This is a major difference compared to vaccines against viral or bacterial antigens as patients with

advanced cancer are often immunosuppressed and not as responsive to immunisation (162,163). Furthermore, most vaccines against infectious diseases are prophylactic and allow the immune system to be prepared already at the onset of infection. This is in sharp contrast to cancer vaccines performed in "late stage" disease when a large number of tumor cells are present. Although there are striking examples of regression of large tumors (137,138), it is likely that vaccines against cancer will operate more efficiently when administrated early during tumor progression or directly after conventional treatment modalities, such as surgery or chemotherapy. One example of this possibility comes from a vaccination study against lymphoma with an idiotype protein vaccine in combination with GM-CSF (164). Eight out of eleven patients that were in their first clinical complete remission after standard chemotherapy but still had molecular signs of disease, as determined by the presence of cells with the 14;18 translocation, went into molecular remission upon vaccination. Thus, the immune system efficiently eradicated the minimal residual disease. In situations of larger tumor burden, adoptive transfer of high numbers of T or NK cells may be needed. The use of different strategies depending on the tumor burden is illustrated in **figure 7**.

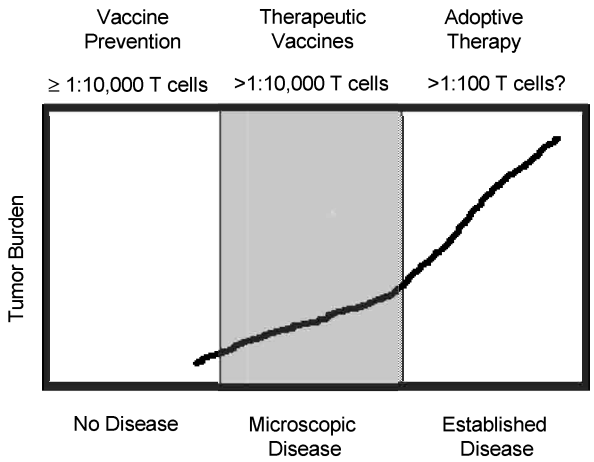

Figure 7. Schematic figure of different approaches of immunotherapy and their usefulness in different stages of disease (adapted from a talk of Nora Disis with her kind permission).

Bone marrow transplantation (BMT) has recently been used as immunotherapy of solid tumors with promising results (165). The intersection of BMT and more specific immunotherapy based on the knowledge of defined antigens is particularly interesting and illustrated by two recent studies. First, Dummer and colleagues exploited the homeostatic proliferation phase of T cells occurring after sub lethal irradiation (conditioning) to induce strong CD8 T cell responses against tumor associated self

antigens (166). This was based on the concept that homeostatic proliferation occurring in situations of lymphopenia, is driven by recognition of self MHC/peptide ligands similar to those that control positive selection of T cells in the thymus (167-169). Adoptively transferred T cells, educated during the homeostatic reconstitution in lymphopenic and tumor bearing animals, grew to become tumor specific. Similarly, Dudley and colleagues recently reported remarkable clinical responses after adoptive transfer of tumor specific, tumor infiltrating lymphocytes (TIL) into melanoma patients that were conditioned with low doses of chemotherapy (170). Around 50% of the patients experienced objective clinical responses. The reasons for the beneficial effect of conditioning the patients remain unclear. It may involve the presence of homeostatic proliferation, but other factors such as depletion of immunoregulatory $CD4^+CD25^+$ T cells may also contribute.

In another study, T cell clones specific for the Mart-1/Melan A or the gp100 melanoma differentiation antigens were repeatedly infused into patients with metatstatic melanoma along with low doses of IL-2 (171). Objective tumor responses were seen along with preferential localization of the infused T cells to the tumor sites. It has been shown that adoptively transferred tumor specific T cells are rapidly eliminated from the circulation and that the effect of adoptive immunotherapy therefore is short-lived (172,173). This may be due to homeostatic forces contracting the lymphocyte population to normal levels. Alternatively, "*in vitro*" expanded T cells may have a limited lifespan due to lymphocyte senescence (174). In other models, EBV specific T cells were shown to have the potential of long-term survival when adoptively transferred to a recipient (175). This property was recently exploited by expressing a chimeric tumor specific TCR in EBV specific T cells (176). These T cells survived long-term *"in vivo"* and provided a source of T cells capable of killing both EBV infected cells and tumor cells expressing the antigen recognized by the chimeric receptor.

It will be important to define the factors that influence the functional properties and the survival of *"in vitro"* expanded T cells to be used for adoptive immunotherapy against cancer.

Immune escape mechanisms

As immunotherapy becomes more efficient, the immunological selection pressure against tumor cells will increase. In combination with the inherited genetic instability of tumor cells, this will lead to the development of immune escape variants (177). Below I will describe several ways by which tumors may escape the immune system. The list of immune escape mechanisms included here is far from complete but sets the stage for the discussion of my work in the latter part of the thesis. I have chosen to include a rather extensive discussion of tumor induced immunosuppression. I view this as an immune escape mechanism although it is perhaps more a reflection of the large tumor burden in "late stage" disease.

Loss of antigen processing and presentation

As described earlier in this introduction, the presentation of antigens in the context of HLA molecules is crucial both during T cell priming and during the effector phase of an adaptive immune response. Alterations in antigen processing and presentation are

commonly seen both during viral infection and in malignancies. Viruses have adapted to the immune mediated selection pressure in numerous ingenious ways, affecting almost every part of the biochemistry of the MHC presentation pathway (178). For instance, Epstein-Barr viruses (EBV) express a protein (EBNA-1) that carries a repeated sequence (Aly-Gly repeat) that makes it inaccessible for proteasomal degradation into peptides (179). The Herpes simplex virus (HSV) derived protein ICP47 and the cytomegalovirus (CMV) derived protein US6 interfere with TAP and prevent peptide translocation into the ER (180-182).

In tumors, the antigen presentation pathway is disrupted as a consequence of mutations and / or deletions of one or several genes encoding for components of the antigen processing machinery. Complete HLA loss is a common event in several murine and human tumors (183-185). Total loss of HLA class I is usually associated with alteration of β_2m expression as the MHC class I molecule is not properly assembled in the absence of this molecule. β_2m mutations along with loss of heterozygosity (LOH) of the second allele has been described and may be associated with progression of disease in the course of adoptive immunotherapy (186,187). However, also the loss of proteasomal subunits such as LMP-2 and LMP-7 as well as of peptide transporters TAP1 and TAP2 leads to total loss of HLA class I and was reported in several different tumor types including cervical carcinoma, small cell lung carcinoma, non-small cell lung cancer, prostate carcinoma and renal cell carcinoma (188-192). Tumors often display a selective loss of HLA-haplotype, locus or allele. Haplotype losses may be the result of LOH on chromosome 6 (193) whereas several mechanisms may account for the locus down-regulation. Sequential loss of several alleles in successive metastasis from a single patient who underwent immunotherapy suggests that immune mediated selection pressure plays a pivotal role in selecting the HLA loss variants (194). In melanoma, HLA-B locus down-regulation was shown to correlate with c-myc over-expression (195) and in colon carcinoma the same phenotype appeared as a result of decreased binding of transcription factors to locus specific regulatory elements (196). A study of several melanoma cell lines demonstrated low or no expression of gene products of the HLA-C locus (197).

An interesting aspect of the loss of certain HLA alleles comes from the study of Ikeda et. al. where they demonstrate that tumor cells that had lost HLA-Cw upon progression could be sensitized to tumor antigen specific CTLs (198). These CTLs were incapable of killing tumors derived from an earlier biopsy, before any loss of HLA had occurred, although they expressed the same tumor antigen. The reason turned out to be that the expression of HLA-Cw by early tumors enabled ligation of HLA-Cw specific KIRs, expressed by the CTLs. Thus, loss of HLA resulted in increased sensitivity to CTLs. Similarly, tumor cells that lose HLA class I should become more sensitive to NK cell mediated lysis because they fulfill the criteria of "the missing self hypothesis". Indeed, this has been reported to be the case. Loss of β_2m leads to increased NK sensitivity and effective tumor elimination (199,200). However, NK cells may express iNKR belonging to different families that only partially overlap and the failure of one receptor-ligand system may be compensated by another i.e., lack of HLA-C : KIR signaling may be compensated by the HLA-E : CD94/NKG2C interaction (201). Furthermore, NK cells are dependent also on activation signals, which is why lack of inhibition is not always enough. I will discuss the balance of activating and inhibitory signals to NK and T cells below and in relation to **paper I** and **II**.

Antigenic loss variants

An unfortunate consequence of efficient immunotherapy is that the immune system selects for tumor variants that are no longer recognized, i.e., that have lost the targeted antigen. Antigenic variation is the hallmark of many successful pathogens and may be the most difficult obstacle for the induction of long lasting immunity against many infectious diseases including HIV (202). This phenomenon was beautifully demonstrated in the study of Yee et al. where the histology of recurrent metastasis following adoptive transfer of mart-1 specific T cells was examined (171). In three out of five studied patients, the recurrent metastasis lacked expression of Mart-1 while expression of gp100 and tyrosinase remained intact. Similar observations has been made in the course of peptide and DC immunisations (203-205). These results strongly suggest that one has to target multiple tumor antigens, or perhaps antigens that are crucial for the growth of the tumor cells. In the advent of antigen loss variants the tumor cells would stop dividing and thereby lose their malignant potential.

The Fas "counter attack"

The Fas "counter attack" refers to the possibility that tumor cells attack and kill T cells that home to the tumor. This concept was based on the observation that FasL expressing sertoli cells were not rejected when transplanted into allogeneic mice (206). It was concluded that FasL expression was a key feature of immunological privilege supported by the finding of FasL expression in the eye (207). This held great promises for future clinical applications as FasL expressing transplants would be tolerated by the recipient. The first example of FasL expression on tumors was on colon cancer, rapidly followed by studies of melanoma (208,209). FasL expression had functional consequences as it induced apoptosis of Fas expressing Jurkat T cells and tumor infiltrating lymphocytes. FasL expression was also reported on ovarian carcinoma leading to signaling defects and apoptosis of tumor associated lymphocytes (210).

More recently, the concept of a tumor mediated counterattack has been revisited (211). When trying to transplant FasL expressing islet β-cells, researchers observed granulocyte infiltration and a rapid rejection of the transplant (212); hence the opposite to tolerance. Similar observations has been made in tumor models in which FasL expressing tumors were rapidly rejected (213-215). Furthermore, several researchers failed to see expression of FasL on tumors previously reported to express this molecule (216,217). The Fas mediated apoptosis of infiltrating lymphocytes seemed rather to be the consequence of T cell activation and activation induced cell death (218).

The Fas death receptor signaling pathway may instead be a very important component of host defence against tumors (219). Not surprisingly, escape variants that are resistant to death receptor signaling have evolved (see below).

Escaping death receptor signaling

Two death receptor signaling systems that contribute to the immune surveillance of tumors are FasL and TNF related apoptosis inducing ligand (TRAIL) (219-222). Death receptors have cytoplasmic sequences, death domains, which transmit the apoptotic signal upon ligation. This triggers a cascade of caspases involving caspase 8 (also known as FLICE), caspase 3, caspase 6 and 7. Tumor cells may become resistant to death receptor signaling by expressing an inhibitor of caspase 8, cellular FLICE inhibitory protein (FLIP) (223-225). Over-expression of FLIP was shown to mediate tumor cell escape "*in vivo*" (224). Tumors also display loss of Fas expression which

renders them resistant to apoptosis (226-228). Alterations may appear further downstream in the death receptor signaling pathway, including functional impairment of FADD and caspase 10 by inactivation mutations (229,230). Similarly, tumors escape death receptor signaling by losing the expression of TRAIL receptors or downstream signaling components (231).

Yet another intriguing way for tumors to escape recognition by CTLs and NK cells is by over-expressing PI-9 (in mice known as SPI-6) (232). PI-9 is a serine protease inhibitor that inactivates granzyme B which is one of the important components of granule mediated CTL killing (233). One suggested physiological role of SPI-6 is to protect activated dendritic cells from being killed by the induced CTLs during a Th1 response (234).

Engaging inhibition - Blocking activation

The balance between activation and inhibition of T and NK cells is dependent on the quality of a number of receptor-ligand interactions. Evidence suggests that tumors evade T and NK cell recognition by shifting the balance towards inhibition. Ectopic expression of the HLA-G molecule has been described in several different tumors including melanoma, renal cell carcinoma, breast carcinoma, cutaneous lymphoma and ovarian carcinoma (60-62). HLA-G expression protects tumor cells from lysis by T and NK cells via ligation of inhibitory receptors LIR-1 and/or KIR2DL4 and indirectly via the HLA-E CD94/NKG2A interaction as described earlier in this introduction (**figure 6** and refs. 66-68,70). HLA-G also shuts down other effector functions such as the production of IFN-γ and Fcγ receptor mediated activation of LIR-1 expressing macrophages (235-237). Soluble HLA-G1 was furthermore demonstrated to induce apoptosis of $CD8^+$ T cells via a Fas-FasL dependent mechanism (238), perhaps constituting a refined version of the counter attack concept. The secreted form of HLA-G1 induced the expression of functional FasL in activated T cells and this was shown to be the result of the interaction between the conserved α3 domain of the HLA-G1 molecule and the CD8 molecule. Similarly, soluble HLA class I molecules, including the non-classical HLA-E and HLA-F molecules, as well as alleles of HLA-C, were shown to induce apoptosis of NK cells upon ligation of activating receptors (239). Again, this was dependent on the up-regulation of FasL in stimulated NK cells resulting in suicide along with killing of neighboring Fas expressing NK cells (Fratricide). An alternative, but closely related way for tumors to avoid being killed by NK and T cells is by shedding decoy ligands for activating receptors. Tumors were found to secrete soluble MICA, which is the ligand for the activatory receptor NKG2D (240). It was demonstrated that soluble MICA is frequently found in sera of cancer patients at levels sufficient to cause down-regulation of NKG2D on tumor infiltrating and circulating αβ- and γδ T cells and NK cells. As NKG2D is involved in direct recognition of MICA expressing tumors as well as in co-stimulating T cell responses this had functional consequences, down-modulating the reactivity and cytokine production capacity of anti-tumor CTLs.

Suppression of anti-tumor responses by regulatory T cells

Recently, a subset of T cells, co-expressing CD4 and CD25 (IL-2R α-chain), with unique immunoregulatory properties was described (241). These regulatory T cells (T_{reg}) seem to play a pivotal role in immune homeostasis, protecting the host from several T cell mediated autoimmune disorders including Type I Diabetes (242),

hypothyroidism due to thyroiditis (243), and pernicious anemia due to gastritis (244). $CD4^+CD25^+$ regulatory T cells dramatically suppress the function and proliferation of $CD4^+CD25^-$ and $CD8^+$ T cells (245). Much of the immunoregulatory function of these cells have been attributed to their capacity to secrete immune suppressive cytokines such as TGF-β and IL-10 which may severely inhibit CTL responses as will be further described below (246,247). Although beneficial in protecting the host from autoimmune disorder, regulatory T cells may also dampen anti-tumor responses. When T_{reg} were depleted in mice, transplantable tumors were efficiently rejected by the host immune system (248,249). T_{reg} has been shown to infiltrate several types of human tumors, including non-small cell lung cancer, pancreas, breast and ovarian carcinoma (250,251). Regulatory T cells are also found at a higher frequency in the peripheral blood of cancer patients and may induce peripheral ignorance of tumor cells facilitating metastatic spread of the disease (251). It should be stressed that there is a large amount of "early" literature describing similar immune suppression inferred by the later banned "suppressor T cells" and it remains to be seen whether or not these T cell subsets overlap.

Tumor induced immune suppression

Mice with experimental tumors and patients with cancer show evidence of decreased immunological potency (163). This is manifested by decreased proliferation, decreased Th1 cytokine production and a poor cytolytic capacity of TIL and PBL (252-254). On a molecular level this is associated with decreased levels of signaling molecules such as CD3 zeta, $p56^{lck}$, $p59^{fyn}$, ZAP70, JAK3, STAT5 and block of nuclear factor-κB (255-265). The underlying mechanisms for immune dysfunction in cancer patients are probably multifactorial. A large number of factors, including old age (174,266) and cachexia, contribute to the poorly functioning immune system in cancer patients. However, the role of the tumor itself was nicely demonstrated in mice with fibrosarcoma (267). Upon inoculation with tumors, these mice became immunosuppressed as manifested by impaired anti-tumor reactivity of T cells associated with decreased expression of signaling molecules. This phenotype was completely reverted following surgical removal of the tumor, unmasking a population of primed T cells that were capable of rejecting a subsequent tumor challenge.

Several mechanisms may account for the tumor induced immune suppression. In the mid seventies macrophages from tumor bearing hosts were found to inhibit proliferative responses of splenocytes in a non-antigen dependent manner (268). Splenocytes from tumor bearing individuals had proliferative responses that were 90 percent below normal. However, if these "suppressor macrophages" were removed, the proliferation was completely restored. Adoptive transfer of macrophages from tumor bearing mice facilitated tumor growth in the recipient (269). Several human tumor types including carcinomas of the breast, bladder and cervix show an association between the levels of infiltration of tumor associated macrophages and poor prognosis (3). Such pro-tumor effects by macrophages may depend on secretion of pro-angiogenic cytokines and enzymes (270), secretion of immune suppressive cytokines (271) and release of free radicals including NO (272) and hydrogen peroxide (273). However, the role of tumor associated macrophages in tumor progression is controversial as there is also evidence for an anti-tumor effect by these cells. Some of the mechanisms behind the dual effect of tumor associated macrophages are summarized in **figure 8**.

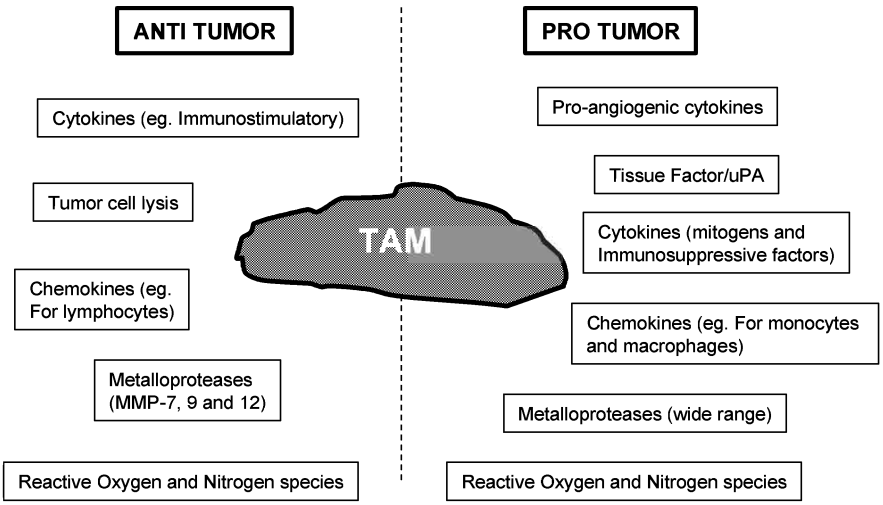

Figure 8. Tumor associated macrophages (TAM) and their dual effects on tumor progression. Adapted from Bingle et. al. (3).

Several early studies suggested that macrophages inhibited T and NK cell function by releasing hydrogen peroxide (H_2O_2) (273-275) and NO (272). The addition of catalase, an enzyme that specifically catalyses the reduction of H_2O_2, rescued NK cells from the inhibitory effects of activated macrophages (273). This was further supported by other investigators showing that histamine, an inhibitor of H_2O_2 production, could rescue NK cells from the detrimental effects of activated macrophages (276-278). Later, it was shown that several other features of tumor induced immune suppression, including loss of Th1 cytokine production, and decreased expression of signaling molecules, were induced by activated or tumor infiltrating macrophages (279,280). These effects could be mimicked by exogenous H_2O_2 and were restored in the presence of catalase. Interestingly, also tumor cells have been found to produce large amounts of hydrogen peroxide (281), which could potentially induce T cell dysfunction of tumor infiltrating T cells. The role of oxidative stress in regulating T cell functions will be further discussed in relation to **paper III** and **IV**.

The unresponsiveness of the immune system in patients has also been attributed to the secretion of immune suppressive cytokines. IL-10 is frequently detected in high amounts in cancer patients (282-285). This cytokine may skew immune responses away from a protective and anti-tumor Th1 response (286-288). IL-10 also inhibits differentiation, maturation and functional status of DC (289), thus interfering with the induction of anti-tumor responses. Furthermore, IL-10 may suppress T cell mediated immunity by down-regulating the function of TAP molecules and the expression of

MHC class I molecules on target cells (290,291). The immune suppressive properties of IL-10 have been adopted by the Epstein-Barr virus which has a gene product (BCRF1) with striking homology to the mammalian IL-10 sequence (292). TGF-β is another cytokine that inhibits activation, proliferation and activity of lymphocytes (293,294). This cytokine is often found at high levels in malignancies and is associated with poor prognosis and lack of response to immunotherapy (295,296). Interestingly, TGF-β promotes the expression of inhibitory CD94/NKG2A receptors on T cells (297), which may allow tumor cell escape from host immunity.

RESULTS AND DISCUSSION

Part 1: Non-classical HLA class I molecules and inhibitory NK receptors

Paradoxical effects of IFN-γ on CTL mediated lysis of tumors

Our interest in the regulatory effects of NKR on T cell mediated anti-tumor responses began with the observation that IFN-γ treated ovarian tumor cells became resistant to the lytic activity of allo- and peptide specific $CD8^+$ T cell clones **(paper I)**. This observation was done when short-term ovarian carcinoma cell lines (OVAC) were established in order to assess their sensitivity to T cells, and relate this to their antigen processing and presentation properties upon cytokine treatment. It turned out that most tumor cells had an intact antigen presentation machinery including components of the immune proteasome: LMP-2, LMP-7 and MECL-1 as well as the chaperon tapasin and the TAP 1 and 2 molecules. These molecules were induced by IFN-γ leading to increased levels of HLA class I molecules at the cell surface. Yet tumor cells became resistant to CTL mediated lysis upon IFN-γ treatment.

Several possible explanations for this observation were excluded. Ovarian carcinomas were previously shown to express functional FasL and could induce apoptosis of TIL (210). As IFN-γ is known to up-regulate the expression of FasL (298), we first investigated whether IFN-γ treated tumors induced T cell death or T cell dysfunction. This was done using two different experimental settings. In "reverse labeling" assays, T cells were labeled with Cr^{51} and untreated or IFN-γ treated tumors were used as effectors. No induction of T cell death could be detected. In a complementary set of experiments, T cells were co-cultured with IFN-γ treated but HLA mismatched tumors. A third party Cr^{51}-labeled cell line was then added (**figure 9**). These experiments revealed that T cells remained perfectly capable of killing the third party cell line despite the presence of IFN-γ treated tumors. Thus, there was no evidence for impaired CTL viability or function as a consequence of direct interaction with IFN-γ treated tumor cells or due to the release of a soluble factor.

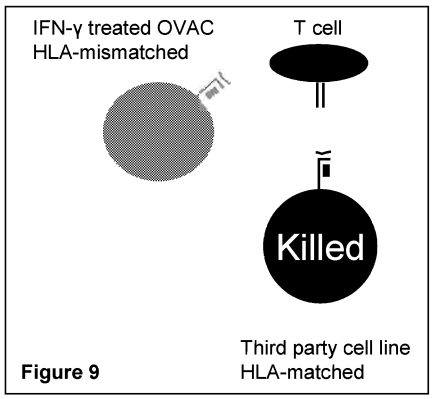

Figure 9

An alternative explanation for the paradoxical protection of OVACs from CTL lysis was that tumor cells became resistant to apoptosis upon IFN-γ treatment. This possibility was investigated in a cell free system using an anti-Fas antibody (CH-11). In agreement with other reports we could demonstrate that tumor cells actually became more sensitive to Fas mediated apoptosis after treatment with IFN-γ (299). This result probably reflected increased expression of Fas receptors on tumor cells and excluded that IFN-γ treated tumor cells had become resistant to apoptosis.

Shifting the balance from activation to inhibition

IFN-γ was previously shown to promote tumor cell resistance to NK cells due to its capacity to induce the expression of class I (300). Tumor cells that express high levels of MHC class I are more tumorigenic in models where NK cells play a pivotal role in host defence against tumors (301). Furthermore IFN-γ treated tumors are resistant to non-MHC restricted lysis by LAK cells (302,303). This protection depends on the molecular interaction of MHC class I molecules and inhibitory NK receptors (38,39,304). It has become clear that inhibitory NK receptors are frequently expressed on activated/memory T cells although the physiological role of such an expression remains elusive (44). There is abundant evidence that antigen specific recognition of virus infected cells and tumors can be inhibited by ligation of several different types of inhibitory receptors (305-307). Thus, the inhibitory signal may over-ride the triggering signal provided via TCR interaction. This was illustrated in a recent study where co-engagement of the TCR and a KIR molecule on tumor specific T cells inhibited early events of T cell activation such as phosphorylation of ZAP70 and LAT, lipid raft polarisation and TCR/CD3 accumulation (308). Results from several model systems suggest that the negative signal dominate over the activating signal when both are present simultaneously (309,310). Thus, it was conceivable that the IFN-γ mediated tumor cell resistance that we observed in our tumor model was due to a shift in the balance of activating and inhibitory signals to the CTLs.

To investigate this possibility we first examined the expression of inhibitory receptors on our CTL lines and clones. As we had observed the IFN-γ mediated protection in a large number of effector-target pairs it seemed unlikely that this phenomenon was due to the recognition of a particular HLA allele by its corresponding inhibitory receptor. Instead, we focused on the CD94/NKG2A receptor as this receptor may survey a broader panel of HLA class I alleles due to its recognition of HLA-E. All T cell clones and lines used in **paper I** were found to express inhibitory CD94/NKG2A receptors and some co-expressed LIR-1 and KIR molecules.

The functional consequences of such NKR expression were evaluated in blocking experiments. By adding an antibody against CD94 in the Cr-release assays we could restore lysis of IFN-γ treated cells (**paper I**). From this, we concluded that IFN-γ treatment of tumor cells shifted the balance from activation to inhibition, turning of the cytolysis of otherwise sensitive cells. Furthermore, we were able to restore the capacity of TAL to kill IFN-γ treated autologous tumor cells, revealing that ligation of CD94/NKG2A was the principle mechanism behind decreased LAK sensitivity of IFN-γ treated tumors (**paper I**).

Expression of non-classical HLA class I molecules

The only known ligand for CD94 is HLA-E which is why we investigated the expression of this non-classical HLA class I molecule on the short-term ovarian carcinomas. This was done using a real-time PCR assay as we could not get access to serological reagents. We could demonstrate spontaneous and IFN-γ inducible mRNA expression of HLA-E in all tested OVACs, but also in several long-term tumor cell lines that were not protected by IFN-γ. Thus, the results from the real-time PCR experiments could not explain the discrepancy between short-term and long-term tumors. We needed to search for alternative explanations to this phenomenon.

HLA-E surface expression can be up-regulated by IFN-γ via a direct effect of transcription (**paper I**, ref. 311). However, it may also be the result of increased

accessibility to leader sequences of classical HLA class I molecules and / or HLA-G. The signaling property of the HLA-E - CD94/NKG2 interaction is highly dependent on the peptide that is bound to the HLA-E molecule. As described earlier in this thesis, certain leader sequences have the capacity to promote strong signaling to CD94/NKG2 receptors (**figure 5**). It was recently shown that stressed cells become susceptible to lysis by CD94/NKG2A expressing NK cells due to binding of a signal sequence peptide of human hsp60 to the HLA-E molecule. This peptide induces expression of HLA-E but does not permit signaling through CD94/NKG2 receptors (75). Such stress induced peptide interference (SPI) would allow infected (stressed) cells to be efficiently removed by T and NK cells despite the expression of inhibitory receptors by these cells.

The non-classical HLA class I molecule HLA-G was of interest to our study as the leader sequence of this molecule promotes a particularly strong signal to CD94/NKG2 receptor bearing cells (**table 2**). Furthermore, ectopic and IFN-γ inducible expression of HLA-G had been reported in several tumor types (60-62). We found frequent expression of the HLA-G molecules at the surface of non-cultured ovarian tumor cells (**paper 1**). Although surface expression of HLA-G was rapidly lost upon "*in vitro*" culturing of the tumor cells, it could still be detected in total cell lysates of the short-term tumor cell lines. Interestingly, HLA-G expression of OVACs could be induced by IFN-γ treatment. Based on this observation we speculated that IFN-γ treatment of tumor cells resulted in increased accessibility to the leader sequence of HLA-G, providing HLA-E with a highly protective peptide ligand (**figure 10**). This model fits with the observation that long-term tumor cell lines were not protected by IFN-γ as none of these cell lines expressed HLA-G.

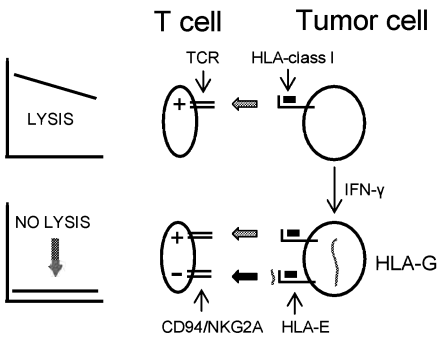

Figure 10, Model of IFN-γ mediated protection of short-term OVAC. Up-regulation of HLA-G provides HLA-E with a protective leader sequence peptide. The negative signal via CD94/NKG2A overrides positive signaling via the TCR.

The correlation between HLA-G expression and the protective effect of cytokine treatment was corroborated when investigating the CTL sensitivity of TNF-α treated OVACs. TNF-α could not induce any expression of HLA-G and did not protect the short-term tumor cell lines from CTLs (Data not included). In fact, TNF-α promoted the CTL lysis of OVACs. In further support for a role of HLA-G we were able to mimic the effect of IFN-γ by exogenously adding a synthetic leader sequence peptide of HLA-G to untreated tumor cells (**paper I**). Similar strategies may be employed by viruses. The cytomegalovirus (CMV) encodes a protein (UL40) that contains a leader sequence with high homology to the HLA class I leader sequences (312,313). This UL40 derived sequence binds the HLA-E molecule and confers protection of virus infected cells from CD94/NKG2A$^+$ NK cells. Interestingly, IFN-γ acts in synergy with UL40 to up-regulate the expression of HLA-E and protect virus infected cells from NK lysis (314).

The finding that HLA-G is expressed by ovarian tumor cells in around 50% of the patients may have practical consequences regardless of the HLA-E - CD94/NKG2 interaction described above. HLA-G was shown to interact with LIR-1 to inhibit T and NK cells directly (315). As LIR-1 was detected on ovarian tumor associated lymphocytes (further discussed below) this interaction may have an additive inhibitory effect on immune recognition of ovarian carcinomas.

The loss of HLA-G surface expression upon "*in vitro*" culture is an intriguing observation previously made by others (62). In fact, tumor cell lines only rarely express HLA-G at the protein level although mRNA expression can sometimes be detected (316). The reason for this observation remains elusive. Perhaps HLA-G expression is driven by cytokines or other soluble factors that are present "*in vivo*" but lacking during "*in vitro*" cultivation. Indeed several cytokines including IFN-γ, IL-10 and GM-CSF have been shown to promote expression of HLA-G. The demonstration of HLA-G expression on macrophages and T cells present in a breast cancer lesion, supports the role of environmental factors for sustained expression of this molecule (61).

A clinically important aspect of HLA-G expression comes from a study of the therapeutic effects of IFN-α2b in melanoma. A retrospective analysis of patients who underwent IFN-α2b therapy showed that tumor samples from patients that did not respond to the treatment expressed HLA-G, but lacked expression of classical HLA class I molecules (317). Another study demonstrated secretion of soluble HLA-G1 which was specifically increased after therapy with IFN-alpha in melanoma patients (318). However, preliminary results from our group indicate that IFN-α treatment does not protect the short-term tumor cell lines from CTL lysis.

Expression of NKR by Tumor Associated Lymphocytes

We investigated the expression of the CD94/NKG2 and LIR-1 receptors on lymphocytes infiltrating the peritoneal cavity of ovarian carcinoma patients (**paper II, figure 11**). Several interesting observations were made. First, we confirmed that CD56$^+$ tumor associated T cells expressed high levels of CD94/NKG2 and LIR-1 as compared to CD56$^-$ T cells. This subset of T cells was previously shown to contain high amounts of perforin and displayed potent cytotoxic activity (319). However, CD56$^+$ T cells are present in the peripheral blood of healthy donors and expresses similar levels of CD94/NKG2 and LIR-1.

The CD94/NKG2 receptors may be activatory or inhibitory depending on the subtype of NKG2 molecule that bind (52). Several antibodies used to detect the CD94 molecule show differential binding patterns depending on the heterodimers that are expressed by the cells (51,320). More specifically, weakly stained cells do not express inhibitory CD94/NKG2A receptors while they may express all activating isotypes including NKG2C, -E and –H (53). In contrast, brightly stained cells, also co-express inhibitory CD94/NKG2A receptors. Importantly, this has functional consequences. Brightly stained cells are inhibited regardless of concurrent expression of activating subtypes of CD94/NKG2, while weakly stained cells are activated upon ligation of the CD94 receptors (53).

When investigating the expression of CD94 on TAL, a remarkable observation was made. Nine out of thirteen samples of tumor associated $CD56^+$ T cells predominantly stained bright for CD94 while none out of eleven samples of this T cell subset derived from patient (n = 5) or healthy donor PBLs (n=6) displayed a similar staining pattern (**paper II**). In contrast, these cells often exclusively expressed activating receptors as determined by the dominance of weakly stained cells. A similar observation was made in melanoma where metastatic lesions contained more of the inhibitory NKG2A/B subtypes as compared to the primary lesion (321). The reason for this biased expression of inhibitory CD94/NKG2 heterodimers by TAL remains elusive. It may be the result of repetitive TCR stimulation (53) or the influence of cytokines such as IL-12, IL-15 or TGF-β, previously shown to mediate expression of CD94/NKG2A receptors (297,322,323).

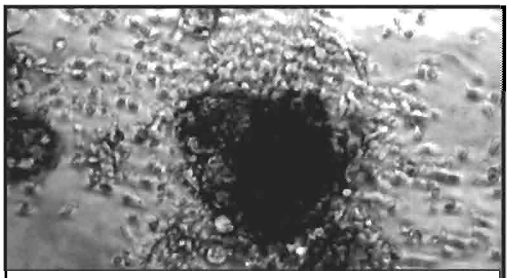

Figure 11, Tumor Associated Lymphocytes surrounding autologous tumor cells in mixt lymphocyte and tumor cell cultures.

In order to perform functional assays a short period of IL-2 mediated expansion of the tumor associated lymphocytes was required. After a two to four week expansion period, expanded TALs efficiently killed autologous tumors (**paper I**). This killing was dramatically reduced by IFN-γ but could be restored in the presence of an anti-CD94 mAb. These results clearly indicate that the expression of CD94/NKG2A by tumor associated lymphocytes severely affect their capacity to recognize and eradicate autologous tumors. The complementary ligand-receptor system, HLA-G / LIR-1, may also contribute to the silencing of anti-tumor T cells "*in vivo*".

A race against time – Physiological considerations

IFN-γ was previously reported to be expressed in ovarian cancer biopsies (282). Furthermore we have detected high amounts of IFN-γ in the ascites fluid of several patients with ovarian carcinoma (Data not included). Perhaps the treatment of tumor cell lines with IFN-γ induces a tumor cell resistance that merely reflects the physiological situation in the host? A plausible scenario can be deduced from the findings of Moser et al (324). They described that polyoma virus specific T cells rapidly up-regulated the expression of CD94/NKG2A upon recognition of viral antigens. This had functional consequences as $CD94/NKG2A^+$ T cells were unable to

control virus induced oncogenesis in susceptible mice (Moser et al). It was suggested that components of innate immunity as well as virus specific T cells may secrete IFN-γ early in the course of an infection, which alters the balance of iNKR and TCR signaling due to increased levels of inhibitory ligands such as Qa-1 (the murine ortholog to HLA-E). Similarly, NK cells or antigen specific Th1 cells can be triggered by tumors to secrete IFN-γ, leading to an unwanted negative feedback loop, turning off $iNKR^+$ T and NK cells. One of the major functions of NK cells is to secrete IFN-γ. Nevertheless, IFN-γ production by NK cells dramatically decreases their chances to efficiently kill surrounding targets because of the increased expression of ligands for iNKRs. Lewis Lanier, one leading NK researcher, described this situation as "a race" (personal communication). The NK cell simply has to kill its target before IFN-γ exerts its function. Our results suggest that this scenario, depicted in **figure 12**, may also be relevant for T cells. Therefore, the use of exogenous IFN-γ administration in immunotherapy against tumors needs to be carefully considered.

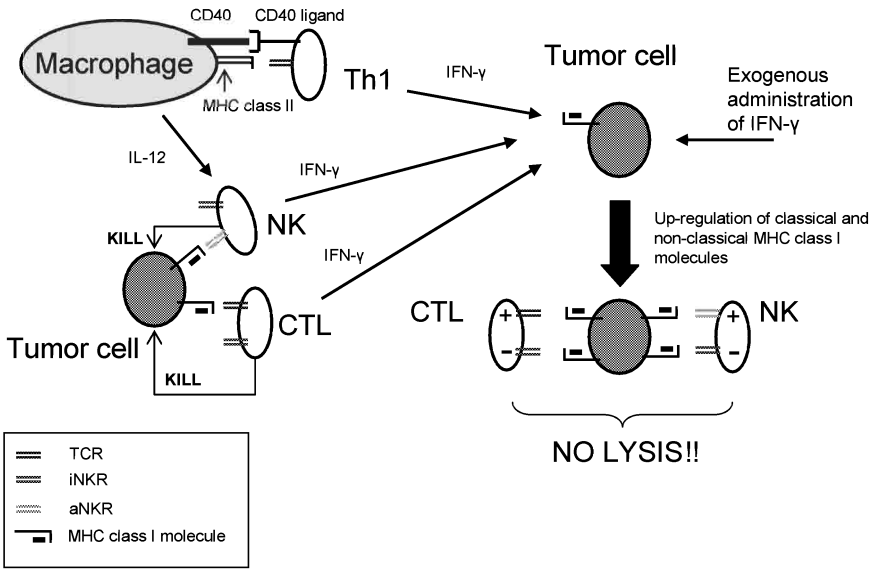

Figure 12. Model for IFN-γ mediated inhibition of anti-tumor NK cells and CTLs. Tumor-infiltrating macrophages present MHC class II:tumor peptide ligands to antigen-specific Th1 cells and induce them to produce IFN-γ. In addition, by upregulating CD40 ligand, these activated Th1 cells ligate CD40 receptors on the macrophages and induce them to secrete IL-12; IL-12 then triggers IFN-γ production by NK cells. Low MHC class I expressing tumor cells also activate NK cells and tumor-specific CTLs to produce IFN-γ. By upregulating classical and non-classical MHC class I surface expression by tumor cells, IFN-γ, either endogenously produced or injected, promotes ligation of iNKRs on NK cells and anti-tumor CTLs and turns off their cytotoxic effector function. (iNKR and aNKR; inhibitory and activatory NKR respectively) Adapted from Aron Lukacher (5).

Over-representation of regulatory NK cells in ascites

Two phenotypically and functionally distinct NK cell subsets have been identified depending on their surface expression of the adhesion molecule CD56 (325). Around 90% of human NK cells are $CD56^{dim}$ (326). This subset of NK cells is highly cytotoxic and expresses FcγRIII (CD16). The remaining ten percent are $CD56^{bright}$ and are often referred to as regulatory NK cells based on their superior capacity to produce a number of cytokines, including IFN-γ, TNF-α and β, IL-10, IL-13 and GM-CSF (327-329). Regulatory, $CD56^{bright}$ NK cells have a comparably low cytotoxic potential. The two subsets also display different patterns of NKR expression (329). While $CD56^{dim}$ NK cells express KIRs and LIRs, the $CD56^{bright}$ cells predominantly express CD94/NKG2 receptors. The role that each of the two subsets play under physiological and disease conditions, is still unclear. Expansion of $CD56^{bright}$ cells has been observed in the uterus of pregnant women and was recently shown to depend on the production of Monocyte Inflammatory Protein 1α (MIP-1α) (330).

We observed significant over-representation of $CD56^{bright}$ NK cells in ascites of ovarian carcinoma patients (**paper II**). This NK cell subset represented on average 26% of the NK cells in ascites as compared to 7 and 9% in PBL of patients and healthy donors respectively. Furthermore we observed that the $CD56^{bright}$ NK cells predominantly expressed the inhibitory form of the CD94/NKG2 heterodimer similarly to the $CD56^{+}$ T cells derived from ascites. However, $CD56^{bright}$ NK cells in PBL from healthy donors and patients also exhibited a preferential usage of CD94/NKG2A receptors arguing that this is a general property of this NK cell subset. Indeed, one can find similar observations in earlier publications although no one has stressed this feature as a major point (326,331).

The demonstration of regulatory NK cells in ascites of ovarian carcinoma patients may be of biological significance. Due to their capacity to produce IFN-γ it can be speculated that they could contribute to the negative feedback loop described above and turn off the anti-tumor activity of CTLs.

Part II: Oxidative stress and Antioxidants

Our research group became interested in the role of oxidative stress in tumor immunity several years ago with the observation that T cell dysfunction, induced by tumor infiltrating macrophages, was completely and specifically reverted in the presence of Catalase (279), an enzyme that catalyzes the reduction of H_2O_2 to H_2O + O_2. Thus the tumor induced immune dysfunction inferred by tumor infiltrating macrophages could be largely explained by the capacity of these cells to release high amounts of free radicals. It is important to stress that the action of H_2O_2 may be beneficial to the host as a component of innate immunity against bacteria and other pathogens (332,333). There is also abundant evidence for a potent pro-apoptotic and anti-tumor role of free radicals (334). Furthermore, radiation therapy is dependent on the generation of free radicals and their capacity to induce DNA damage and apoptosis of tumor cells (335).

The way I have come to look at free radicals and their potential role in immunotherapy against cancer is from a biased perspective where the function of the immune system is the main focus. From this point of view it seems obvious that free radicals are harmful and may be responsible for the poorly functioning immune system in cancer patients. It is not my belief that one may cure cancer by altering the redox status in patients with malignancies. However, it may be very important to relieve patients from oxidative stress in order to make them responsive to immunotherapy with the ultimate goal of curing their disease.

Selective targeting of activated / memory T cells

With the advent of new technologies such as the ELISPOT and protocols for intracellular cytokine staining, it became possible to study cytokine release at a single cell level. Furthermore, intracellular staining protocols enabled the simultaneous analysis of cell surface markers to distinguish different subset of cells. Using these techniques I started my PhD investigating the functional consequences of exogenously added hydrogen peroxide to freshly isolated and non-cultured PBL. Several interesting observations were made. We found that H_2O_2 selectively targeted Th1 cytokine production in $CD45RO^+$ memory/activated T cells (**paper III**). The loss of Th1 cytokine production in $CD45RO^+$ T cells was associated with a block of NF-κB activation (**paper III**). This suggested that H_2O_2 inhibited proximal signaling events in $CD45RO^+$ T cells, leading to diminished activation of NF-κB and impaired production of cytokines regulated by this transcription factor (336). The reason for such selective targeting of $CD45RO^+$ cells may be related to the role of the CD45 molecule in T cell receptor signaling (Schematic overview in **figure 13a**). CD45 is a protein tyrosine phosphatase (PTP) that preferentially removes phosphates bound to regulatory tyrosine residues (C-terminal tyrosine residues) on src family kinases. This is essential for T cell activation and sustained TCR signaling as it allows lck and fyn to be phosphorylated at their active sites (337-339). CD45 exists as several isoforms due to alternative splicing of three consecutive exons (4, 5 and 6, designated A, B and C) in the extracellular domain (337). Naïve T cells predominantly express the larger CD45RA isoforms and, over the course of three to five days after activation, they switch to express the smallest CD45RO isoform (340,341). Compatible with the rapid onset of a memory response, $CD45RO^+$ CD4 and CD8 T cells are highly sensitive to antigen and secrete a broad range of cytokines upon stimulation (342,343).

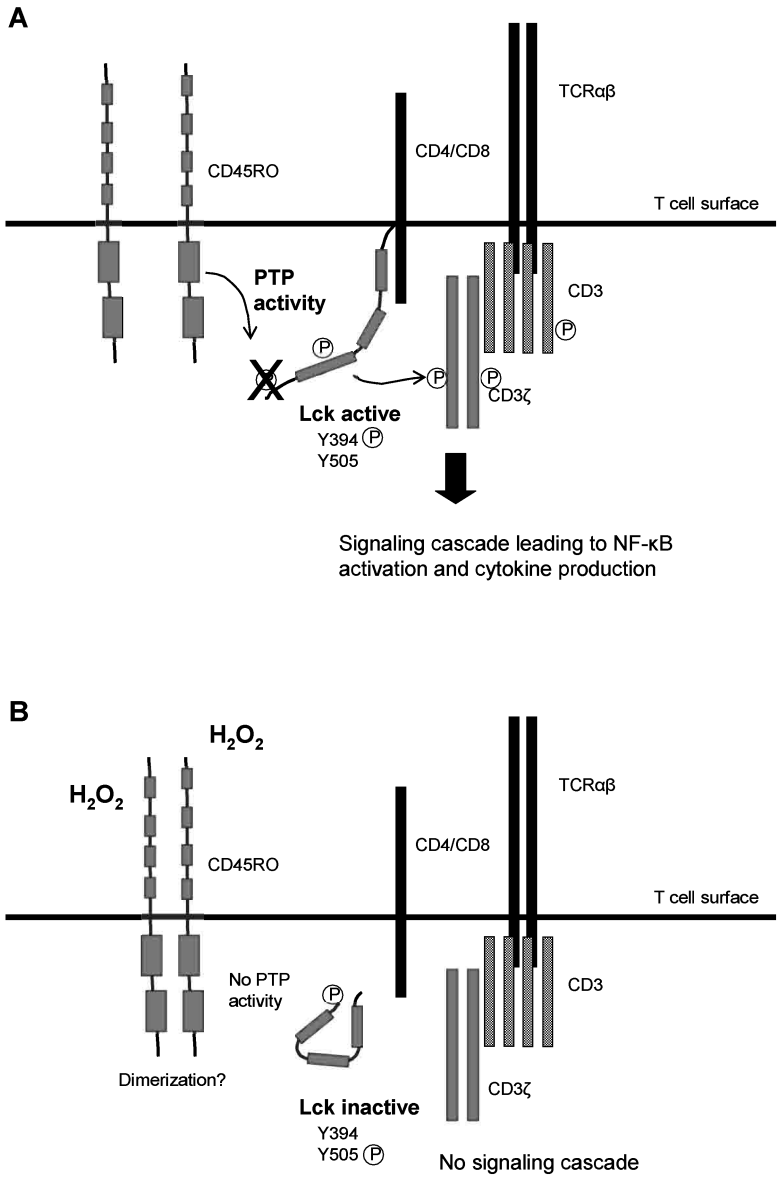

Figure 13. Schematic overview showing the role of CD45RO in TCR signaling. **a**) CD45 acts as a PTP removing phosphates from the tyrosine kinase lck. By removing the regulatory, C-terminal phosphate (Y505), lck can maintain an open configuration and propagate the signaling cascade by phosphorylating CD3ζ, ZAP70 and other signaling molecules. **b**) Hydrogen peroxide (H_2O_2) inhibits the PTP activity of CD45RO and thereby turns off the signalling cascade.

Interestingly, this responsiveness to antigen correlates with the co-localisation of the CD45RO isoform with CD4 / CD8 and the TCR at the cell surface (344-347). The association of the CD45RO isoform with other signaling molecules may be responsible for the more efficient response to antigen by memory T cells. However, it may also explain the enhanced sensitivity of this subset to H_2O_2 (**figure 13b**). It is well known that H_2O_2 functions as a PTP inhibitor (348,349). It can therefore be speculated that H_2O_2 interferes with proper TCR signaling, by blocking the PTP activity of CD45RO. Consequently NF-κB activation and cytokine production is blocked. Such inhibition of CD45 PTP activity would have less functional consequence for $CD45RA^+$ cells, as the CD45RA molecule is less active in promoting TCR signaling due to its distant localization from the T cell receptor (344).

Another recently described phenomenon that may help in understanding the selective targeting of memory/activated T cells by oxidative stress is the enhanced efficiency by which the CD45RO molecule forms inactive homodimers (350). The relevance of this finding to our results becomes obvious taking into consideration the fact that H_2O_2-mediated inhibition of PTP activity is known to involve formation and stabilization of homodimers (351).

It should be stressed though, that the H_2O_2 mediated inhibition of other PTP:s in T cells may instead directly promote kinase activity and induce signaling (352). Indeed, there is a renewed interest in the activity of H_2O_2 as a second messenger. For instance, H_2O_2 may mimic the effects of an antigen, leading to stimulation of resting B cells (353,354). As BCR engagement leads to H_2O_2 production stimulating neighboring BCR in an antigen independent, paracrine fashion, the author suggested that this results in a rapid amplification of the BCR signal (354). It was proposed that the oxidative burst by macrophages serves the sole purpose of stimulating T and B cells. Along these lines, several papers have reported that hydrogen peroxide activate NF-κB in lymphocytes and induce production of IL-2 (355,356). Of note, and as stressed in **paper III**, most studies have been performed on the Jurkat T cell line. In our experiments performed on freshly isolated lymphocytes, no activation of lymphocytes was observed regardless of the duration or level of exposure to H_2O_2. The consequences of H_2O_2 exposure for T cell activation may be cell type dependent and / or relate to the state of activation of the targeted cells.

Differential effect on production of IL-10 versus Th1 cytokines

A surprising finding of the studies presented in **paper III** was that although Th1 cytokine production was reduced by oxidative stress it did not lead to a shift towards a Th2 response. In contrast, production of IL-10 was even more efficiently targeted by hydrogen peroxide. The emerging picture becomes complex as IL-10 can potentially suppress Th1 cytokine production (286,289). By reducing the amounts of IL-10 the Th1 brake is turned off, potentially lading to a more vigorous Th1 response. These mechanisms were obviously not operating during the short time periods over which we examined the cells "*in vitro*" and it is difficult to extrapolate the sequence of events "*in vivo*". The hypo-responsiveness that we observed differs from classical tolerance where IL-10 production is enhanced at the expense of Th1 cytokine production (357-359). On the other hand there is evidence for enhanced T cell function in the presence of IL-10 (360).

Furthermore, IL-10 production seemed to be targeted via a different pathway as the loss of IL-10 production occurred at doses where NF-κB remained unaffected. It

therefore seemed unlikely that this transcription factor was involved. In keeping with this speculation, studies have demonstrated IL-10 production to be mediated by STAT-3 and not by NF-κB (361,362). Another striking difference was that IL-10 production was equally sensitive in naïve and memory / activated T cells. This is compatible with targeting of a pathway that is independent of decreased PTP activity by CD45RO.

Induction of systemic immune suppression?

A central question to any "*in vitro*" study similar to the one performed in **paper III** is the physiological relevance of the model system that is used. Are the concentrations of H_2O_2 representative to the ones present during the cellular interaction between macrophages / granulocytes and T cells? We have recently set up a fluorescence based technique that enables direct measurement of H_2O_2 levels. By using this method we have demonstrated that activated macrophages and granulocytes release doses of hydrogen peroxide that are sufficient to suppress T cell function (25-50μM). Interestingly, preliminary data also shows that granulocytes derived from PBL of colorectal cancer patients release significant amounts of H_2O_2 spontaneously, indicating a constant activation of these cells in the peripheral circulation of cancer patients (Mikael Hansson et al, unpublished observation). If these data are corroborated they provide a molecular support for the hypothesis that activated granulocytes play a major role in systemic immune suppression of cancer patients (363). In the study of Schmielau et al., the existence of activated and H_2O_2-secreting granulocytes in PBL correlated with functional and molecular defects in peripheral T cells of patients with advanced cancer. This indicates that these mechanisms may not only operate in the tumor environment but may also be responsible for induction of systemic immune suppression.

The release of free radicals by granulocytes may be the principal mechanism for generating a state of persistent oxidative stress in colorectal cancer patients. For instance, it was shown that this patient group has lower serum concentrations of free radical scavengers such as vitamin E and C along with increased levels of 8-oxoGua, which serves as a DNA marker for oxidative stress (364). Excluding the unlikely explanation that universally, patients and normal individuals had different eating habits, it may be concluded that the decreased levels of scavengers resulted from enhanced consumption by the high levels of oxidative stress present in the patients. Thus, it may be possible to improve the immunological potency in cancer patients by restoring their antioxidant defenses. This possibility was investigated in the pilot clinical trial discussed below.

Substitution therapy with vitamin E

Epidemiological studies on the effect of dietary factors in prevention of cancer have largely centered on fat and vitamin intake. Evidence indicates that an increased intake of fat and red meat is associated with a higher risk of colorectal, breast and prostate cancer (365). In contrast, high consumption of fruits and vegetables is associated with a reduced risk of several cancers including, lung, oral, pancreas, larynx, esophagus, bladder, and stomach. A molecular explanation for this epidemiological observation is lacking, although it is likely that multiple components of the diet are responsible. A role for vitamin E in decreasing the incidence of prostate cancer was suggested in a large primary prevention study that is being followed up in the recently

launched SELECT trial (the study of Selenium and Vitamin E cancer prevention trial) (366,367).

Vitamin E is a naturally occurring lipid-soluble antioxidant that acts as a scavenger and terminates the cascade of free radical formation induced by toxic and carcinogenic compounds (368). It is found at high concentrations in membranes of T cells and is essential for normal immune function. Deficiency of vitamin E is associated with increased sensitivity to infection and incidence of tumors (369). Supplementation of high doses of dietary vitamin E improves the compromised immune function in elderly and in patients with HIV infection (370-372). Similar results have been obtained in animal models of ageing mice and rats, with evidence of enhanced proliferative capacity and IL-2 production after intake of vitamin E enriched diets (373,374). The beneficial effects of vitamin E on the immune system of elderly support the "free radical theory of ageing" as postulated by Denham Harman (375). Somewhat dramatically, he suggested that we are "rusting from the inside" as we become older.

We investigated the possibility of enhancing the immune function in patients with advanced (Dukes' C and D) colorectal cancer by reconstituting their antioxidant defenses. This was tested in a pilot clinical trial in which patients were given high doses of dietary vitamin E along with vitamin C and selenium at the recommended daily intake (RDA) doses for a period of 2 weeks. Vitamin C and selenium were included in the treatment regimen to recycle vitamin E to its reduced state and thus allow optimal function of vitamin E (369,376,377). Colorectal cancer patients provided a suitable study group as several features of immune suppression were previously reported in these patients. For instance, they exhibit a decline in signaling transduction molecules of peripheral T cells that is associated with severity of disease (378,379), there is evidence for reduced CD4 counts leading to altered CD4:CD8 ratios (380), and Th1 cytokine production is significantly impaired (253,381). Interestingly, surgical removal of the tumor burden leads to a normalization of the cytokine production capacity indicating that the immune suppression was specifically induced by the tumor (381).

Increased Th1 production after vitamin E treatment

In ten out of twelve treated patients we observed an increase in the number of T cells capable of producing IL-2 in response to PMA/Ionomycin (**paper IV**). A patient was considered to be responsive if the increase in the number of IL-2 producing cells was more than 10%. The enhanced IL-2 production after vitamin E treatment may be the central mechanism behind the immunostimulatory effects of vitamin E. IL-2 can reverse T cell anergy and has a potent capacity to induce T cell proliferation via both auto and paracrine loops acting at the IL-2 receptor expressed by activated T cells (382). Moreover, it may be of particular importance in relation to immunotherapy against cancer as IL-2 therapy has been shown to induce objective clinical responses in around 15-20% of patients with advanced melanoma (138).

The increased IL-2 production as a result of vitamin E treatment was evident in all subsets of T cells analyzed including naïve and memory CD4 and CD8 T cells (**paper IV**). However, we observed a tendency towards a more potent stimulatory effect on naïve ($CD45RA^+$) T cells. This is in agreement with a recent study in old mice showing selective IL-2 induction in naïve T cells after supplementation with vitamin E (383). However, this result raises an interesting paradox of the work presented in this thesis. **Paper III** described a selective targeting of IL-2 production in $CD45RO^+$ cells by oxidative stress. Virtually no effect was seen on naïve T cells. Yet, supplementation of

antioxidants to patients with advanced colorectal cancer preferentially boosted IL-2 production in naïve T cells (**paper IV**). Although the stimulation of naïve T cells by vitamin E was not statistically different from that of memory cells, this tendency suggested that vitamin E may boost immune responses via other mechanisms than by acting as a free radical scavenger. Indeed, several different mechanisms of action for vitamin E have been described, including inhibition of prostaglandin production (384), suppression of FasL expression on T cells and thus protection from activation induced cell death (385). On the other hand, our study revealed that vitamin E enhanced PMA/Ionomycin induced IFN-γ production in memory/activated $CD45RO^+$ CD8 T cells which fits better with the role of vitamin E as a free radical scavenger in relation to the findings in **paper III**.

No statistically significant effect was observed on production of IL-10 or TNF-α. The lack of effect on IL-10 is somewhat surprising as this was the cytokine that was most severely affected by hydrogen peroxide (**paper III**). However, a potential role for vitamin E in shifting the Th1/Th2 balance towards a Th1 response is consistent with a recent study where vitamin E was shown to inhibit the gene expression of IL-4 in peripheral blood T cells (386).

Vitamin E increases the CD4/CD8 ratio

Decreased CD4/CD8 ratios have been associated with immunological dysfunction in patients with AIDS (387) and different types of cancer, including multiple myeloma (388), colorectal carcinoma (380) and Hodgkins disease (389). Increased apoptosis of $CD4^+$ T cells may also contribute to the transient immunodeficiency that follows allogeneic BMT (390). The altered CD4/CD8 ratios in multiple myeloma and colorectal carcinoma correlated with the stage of disease, being more reduced in advanced stages (380,388). Others have demonstrated that TIL display a decreased CD4/CD8 ratio while the balance of lymphocyte subsets remained unaltered in peripheral blood (391).

Evidence from human and mouse studies, suggests that vitamin E increases CD4 counts (387,392). In our study, vitamin E significantly increased the overall CD4/CD8 ratios in colorectal cancer patients. This may be of particular importance for the individuals with CD4 counts and CD4/CD8 ratios much below normal. Indeed, a stratified analysis of the five patients with CD4/CD8 ratios below the reference interval for healthy individuals revealed a significant increase after vitamin E treatment.

$CD4^+$ T helper cells are essential for induction of $CD8^+$ cytolytic T-lymphocyte immunity due to their ability to drive the maturation of DC (393). Th cells operate through up-regulation of CD40L, which then interacts with CD40 on DC to cause DC maturation (394). Subsequent CTL induction by activated DC requires CD80/CD86 on the DC to interact with the CD28 co-stimulatory receptor on $CD8^+$ T cells. There is experimental evidence that peptide based vaccination may become more efficient if tumor antigen specific $CD4^+$ T cell mediated responses are concurrently induced by including HLA class II tumor peptides in the vaccine (395,396). The search for such HLA class II restricted epitopes within previously known and new tumor associated antigens has been intensified during the last couple of years (397,398). Furthermore, CD4 T cells can have a more direct role in eliminating tumors during the effector phase (399). For any of these mechanisms to be efficient, one is dependent on the presence of functional $CD4^+$ T cells. We propose that vitamin E, by inducing Th1 production and restoring CD4 counts, may set the stage for subsequent vaccination against cancer.

SUMMARY OF RESULTS

This thesis describes mechanisms of immune escape by tumor cells. Two principally distinct hurdles for effective tumor eradication have been discussed in this thesis. The role of inhibitory receptors and their ligands in ovarian carcinoma, and the consequences of oxidative stress and antioxidants on T cell function. The main findings are summarized below.

- IFN-γ pre-treatment protected short-term ovarian tumor cell lines (OVAC) from allo and peptide specific CTL lysis despite increased levels of the relevant HLA alleles, or presence of increased concentration of peptide at the cell surface of tumors. This protection was dependent on expression of CD94/NKG2A receptors on T cells and was abolished in the presence of blocking antibodies (**paper I**).
- Expression of HLA-E, the ligand for CD94/NKG2A, was detected at the mRNA level in OVACs and could be further induced by IFN-γ. We also found frequent expression of HLA-G on non-cultured tumor cells present in ascites of patients with advanced ovarian carcinoma. Expression of HLA-G was gradually lost upon "*in vitro*" culture but could be induced by IFN-γ treatment of the OVACs (**paper I**).
- Exogenous supply of the HLA-G leader sequence derived peptide (Gsp), known to bind and stabilize HLA-E, mimicked the effect of IFN-γ on OVACs. This suggested that the HLA-E/Gsp complex may be involved in mediating the protection of IFN-γ treated tumors (**paper I**).
- CD94/NKG2 and LIR-1 receptors were expressed on tumor associated lymphocytes derived from ascites in ovarian cancer patients. These receptors were preferentially expressed on NK cells and $CD56^+$ T cells. Furthermore, there was a bias towards expression of the inhibitory heterodimer, CD94/NKG2A, in TAL as compared to PBL of patients and healthy donors (**paper II**).
- We observed an over-representation of regulatory, $CD56^{bright}$ NK cells in ascites compared to PBL of patients and healthy donors. These cells primarily expressed the inhibitory form of the CD94/NKG2 heterodimer (**paper II**).
- Low doses of hydrogen peroxide severely affected T cell function as determined by their cytokine producing capacity. At certain doses of H_2O_2 this functional impairment was not followed by subsequent apoptosis, indicating that there is a dose window of H_2O_2 that render T cells unresponsive without initiating programmed cell death (**paper III**).
- Th1 and Th2 cytokines are differently regulated in conditions of oxidative stress. Th1 cytokine production was mainly targeted in memory/activated ($CD45RO^+$) T cells, while IL-10 production was lost in all T cell subsets. Furthermore IL-10 production was more sensitive to hydrogen peroxide and was lost at lower doses than required to impair Th1 production (**paper III**).
- Loss of Th1 cytokine production in $CD45RO^+$ T cells correlated with a block of NF-κB activation (**paper III**).
- Vitamin E supplementation to patients with advanced colorectal cancer efficiently restored their serum α-tocopherol levels. This was followed by enhanced capacity to produce the Th1 cytokines: IL-2 and IFN-γ. In contrast, no significant increase in IL-10 production was observed indicating a shift in the Th1/Th2 balance, favoring a Th1 response. Furthermore vitamin E increased the CD4/CD8 ratios in colorectal cancer patients (**paper IV**).

CONCLUDING REMARKS

A better understanding of immune escape mechanisms will help in designing novel and more efficient protocols for immunotherapy. The expression of ligands that have the capacity to turn off the function of immune effector cells could potentially abolish an otherwise effective T or NK cell mediated tumor recognition. Such inhibitory pathways can be targeted. For instance, it has been shown that mouse NK cells that were pre-incubated with mAbs against inhibitory receptors had a more potent anti-tumor effect when adoptively transferred into tumor bearing mice (400). Alternatively, one may select or genetically engineer NK and T cell clones to express less of inhibitory receptors and perhaps more of activating receptors, prior to adoptive transfer. Another interesting possibility would be to target the non-classical HLA class I molecules that are ectopically expressed by tumors. This could be done either by humanized specific antibodies or by generating HLA-G or HLA-E allo-specific T cells. With an increasing knowledge of the receptor-ligand interactions that govern T and NK cell activation it may be possible to augment anti-tumor responses and dampen undesirable reactivity of the immune system.

A central result to this thesis is the IFN-γ mediated protection of tumor cells from CTL mediated lysis (**paper I**). Despite this finding I have tried to stress the importance of IFN-γ in anti-tumor immunity and referred to the work of Robert Schreiber and colleagues as the revival of the immune surveillance concept (105,116). A search on PUBMED (IFN-γ and cancer) performed on the 23rd of December 2002 generated more than 4000 publications. Only a few of these publications describe negative consequences for tumor recognition as imposed by IFN-γ. Thus it is clear that IFN-γ is one of the most important cytokines for generating efficient CTL responses, in part by modulating the immunogenicity of target cells. The data presented in **paper I** represents an exception to this rule and points towards a potential side effect that this cytokine may have in certain situations.

The difference between short- and long-term tumor cell lines observed in this thesis merits further attention. It is obvious that long-term "*in vitro*" culturing of tumor cells require different characteristics of the tumor cells than those needed to proliferate "*in vivo*". I believe that one will have to move even further towards a more physiological situation and perform experiments on untouched tumor cells. Such efforts are currently being made in our laboratory and the result from these experiments will be very valuable.

The complexity of redox regulation of immune responses and the dual properties of hydrogen peroxide as a second messenger and a negative regulator of immune reactivity is interesting. In search for a unified model our group has initiated an attempt to delineate consequences on signal transduction pathways, inspired by the selective targeting of $CD45RO^+$ memory/activated T cells. Perhaps all properties of hydrogen peroxide can be attributed to its capacity to shut down protein tyrosine phosphatases, leading to activation or inhibition of signaling cascades depending on which tyrosine residues are available for the targeted PTP.

The capacity of vitamin E to enhance Th1 cytokine production and improve CD4/CD8 ratios will help to generate strong T cell responses upon more specific immunotherapy in colorectal cancer patients. Although vitamin E may have an effect as primary prevention against cancer, I believe its major advantages will be as an immune adjuvant to cancer vaccines.

Since the description of the first tumor antigens by Pierre Van der Bruggen and Thierry Boon in 1991 (401) we are facing a new era of tumor immunotherapy. Along with a tremendous increase in the understanding of basic principles of immunity and novel technologies, we are now ready to run clinical trials and find answers to several of the questions that remain unresolved. It is my belief that immunotherapy against cancer will be effective. However, as for all currently available treatments for cancer, one will have to define the responsive patient groups (types of cancer), the optimal stage of disease and the ideal combination with other treatment modalities. It is likely that different strategies of immunotherapy will be beneficial under different circumstances.

The treatment of advanced stages of cancer with large tumor burden will probably require adoptive transfer of high numbers of tumor reactive T and NK cells. The promising results from several clinical trials of adoptive immunotherapy including allogeneic bone marrow transplantation (165,170,402) makes this field extremely exciting and I wish to continue research on this topic in the future.

ACKNOWLEDGEMENTS

I whish to thank many people for their invaluable and continuous support:

Rolf Kiessling, My boss and supervisor. For trusting the capacity of your students. For your tolerance to weaknesses. For being present and inspiring in the daily experimental work and yet allow the freedom to work independently. For being a role model in the art of working hard without neglecting the non-scientific parts of life.

Giuseppe Masucci, My co-supervisor. For providing the clinical angle to all our work. For your true passion for immunotherapy and your positive attitude.

ALL MY CO-AUTHORS! It would not have been anything without you.

Georg Klein, For being one of the reasons behind my choice of medical studies and perhaps of cancer research. For the "Georg meetings".

Klas Kärre, For introducing me to molecular immunology via the "missing self hypothesis" during my first year at Medical School 1993. The passion for NK cells is still there…

Klas Wiman, Lazlo Szekely, Ismael Okan and Katja Prokrovskaja for introducing me to laboratory work during the summer of 1994.

Max Petersson, For helping everybody with everything. For contributing with your last minute changes (When otherwise?) to this thesis, and for talking…..

Jelena Levitskaya and Victor Levitsky, "The science family". For you contagious devotion to science. For good collaboration. Victor, for convincing me of the importance of focusing at a time when I had seventeen projects running. Both of you for getting me started.

Raja Choudhury, For improving the English of this thesis and of paper III and for continous encouragement.

Lars Franksson, The first person I met in Klas group 1993. For always being sound and for believing in my dead cells.

Yang Rongcun, I will never forget how right you were: "Kalle, you are only planning...." and "Ascites: Several thesis"

The present NKR group of RK: **Håkan Norell** (Not the least for digging deeper than anyone into the FACS data) and **Mattias Carlsten.**

The present OX STRESS group of RK: **Akihiro, Mikael, Volkan and Max.** *H to the O two!*

All former and present colleagues and friends in the Rolf Kiessling group. For all the ever so important details: Teaching the methods, bringing plastics, answering stupid questions and the telephone, filling liquid nitrogen, lending your pens and reagents, telling jokes, sharing disbelieves and good results, baking cakes. For having patience with my loud voice. Thanks **ALL** of **YOU** for EVERYTHING!!!!

Kalle Söderström and Christina Teixiera de Matos. For sharing your expertise in NK receptors and their ligands

Clinical collaborators, **Kjell Schedvins, Christina Hising, Bengt Glimelius and Jan-Erik Frödin**

Staffan Pauli, Gustaf Gaudernack (For not missing the plane….You showed evidence of Norwegian cross-country skiing lungs), **Hans-Gustaf Ljunggren**

Jonas Sundbäck and Jakob Michaelsson for help with the figures of the crystal structures

Pavel Pisa and his group For your true efforts in trying to make immunotherapy a clinical force against cancer.

Håkan Mellstedt and his group and Tina Dalianis and her group.

Anita Edholm, and all the wonderful personel at MTC, CCK and Radiumhemmet, **Birgitta, Joe, Sören, Agnetha and Evi.**

Magnus Backlund and Dan Grandér for giving me the opportunity to teach in the Biomedical program

My family, For so much more than a thesis

The Nilssons for your open arms

Sumpan for orienteering and cross-country skiing

Automobil peking (xxx Djuren) and especially lagis for making it happen every year.

CSN Could you perhaps forget about the money with this gesture?

Huddinge Universitety Hospital and AT friends. For giving me ambivalence

Mats och Conny för arbetsrummet

Jenny-Ann, Rymden.

This work was supported by grants from:
The Swedish Cancer Society, The cancer Society of Stockholm and the King Gustaf V Jubilee Fund, The Swedish Medical Research Council and the European Community.

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