Chapter 1

General introduction on tumor immunology and scope of the thesis

Pathogenesis of cancer cells: intrinsic factors versus extrinsic control

Extrinsic control: tumor immunology
- Immune surveillance
- Immune editing and subversion
- Immune escape

Scope of the thesis
Pathogenesis of cancer cells: intrinsic factors and extrinsic control

Since 2006, cancer has pushed aside vascular diseases as the leading cause of death of men in the Netherlands resulting in 21,200 patients death/year. Perhaps the most frequently asked question these patients pose us (their doctors), is: why? Why did I develop this? Sometimes we are able to identify clearly influencing factors (like smoking or radiation damage), but in the majority of cases we do not know what exactly happened. Pathophysiologically, there are two ways to answer these questions. Oncologists typically employ a cell intrinsic view and explain to their patients that something went wrong within one cell that eventually led to the outgrow of a complete tumor. Immunologists will agree, but will also take extrinsic control into account. They argue that it is quite common that these transformed cells develop but that the immune system normally recognizes and eliminates such cells. Failure to do so leads to outgrowth of a tumor and hence indicates also a problem of the immune system. In other words, the development of cancer consists of two steps; the first being the intrinsic oncogenic event leading to a transformed cell and the second being the failure of the innate and adaptive immune system to recognize and eliminate the transformed cell.

Regarding the intrinsic factors, the development of a tumor cell is a multistep process of genetic alterations, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads (in a Darwinian fashion) to the progression of a normal cell into a transformed tumor cell. It is generally accepted that six intrinsic factors characterize a tumor cell. Tumor cells (1) provide their own growth signals, (2) ignore growth-inhibitory signals, (3) evade apoptotic signals, (4) have limitless replicative potential, (5) sustain angiogenesis, and (6) are able to invade other tissues and metastasize. All cancers presumably acquire these capabilities. However, the acquisition of these capabilities varies significantly (both mechanistically and chronologically) across the spectrum of cancer. Escape from extrinsic control, in other words, escape from immune surveillance was in 2004 added as a seventh hallmark of cancer.

Extrinsic control: tumor immunology

To protect us against viral and bacterial invaders, the immune system is trained to distinguish between self and non-self. Cancer immune surveillance assumes that the immune system is also able to distinguish between self and transformed-self.

The interaction of tumor cells with the immune system comprehends 3 phases; immune surveillance, immune editing and immune escape. In the first
phase, tumor cells are recognized by the immune system and eliminated. This so-called effective immune surveillance will lead to successful eradication of a pre-malignant disease. However, it is also possible that tumor cells enter into a process of prolonged interaction with the immune system. In this second phase, tumor cells with the most immunogenic phenotype will be eradicated and less immunogenic tumor cells will survive. These non-immunogenic tumor cells will, as a result of continuing immune pressure, change their phenotype further (immune editing). Moreover, these tumor cells can upregulate anti-apoptotic receptors, anti-apoptotic intracellular proteins and they can produce immune suppressive factors, thus subverting the immune system. These sculptured non-immunogenic tumor cells ultimately escape from immune surveillance (phase three).

In conclusion, the immunogenicity of a tumor reflects the immunological environment from which it was derived. Successful immunotherapy requires not only a rewarding recognition of both partners, but also the break-through of the local state of immune tolerance that tumors can create.

**Immune surveillance**

The concept of immune surveillance was first proposed by Burnet in 1957\(^5\). As no direct supporting evidence could be delivered, this hypothesis was challenged for a long period of time\(^6;7\). Finally, the idea turned out to be applicable at least for virally induced tumors\(^8\). Immune-compromised humans suffer mostly from virally induced tumors like EBV (Epstein Barr Virus) associated lymphomas and herpes virus associated Kaposi sarcoma’s\(^9\). Renewed interest in immune surveillance came from studies showing that manipulation of important effector mechanisms (IFNγ (interferon gamma) and perforin) of the immune system regulates tumor development and rejection\(^10;11\). More evidence was provided by the observation that mice lacking components of both the adaptive and the innate immune system (such as RAG2 (recombinant activating genes) deficiency and TRAIL (TNF-related apoptosis inducing ligand) deficiency) develop tumors\(^12\).

Cells playing a role in tumor immune surveillance can be either part of the innate or the adaptive immune system\(^13\). The **innate** immune system provides the first line of immediate defense and comprehends of natural killer (NK) cells, γδ T cells, monocytes, macrophages, neutrophils and dendritic cells. Presumably, NK cells are the most important cells of the innate immune system that play a role in tumor immune surveillance. NK cells can detect changes in self molecules (i.e., tumor cells) in the absence of inflammatory signals. Loss of MHC class I molecules was the first NK cell activating signal recognized\(^14\). MHC class I molecules act as an inhibitory signal for NK cells, thus making MHC class I lacking tumor cells a target for NK cells. However, NK cells do not kill all MHC class I missing cells (for example erythrocytes). Further research revealed several activating receptors (the most important being NKG2D)\(^15;16\) and inhibitory receptors (killer inhibitory
receptor, KIR’s) on the NK cell surface. It has now become clear that the activation of NK cells relies on a balance of activating and inhibitory signals. NK cells use their large repertoire of inhibitory and activating receptors to detect the transformed-self. Many tumor cells express activating NK ligands (like MICA and MICB) recognized by NKG2D and are thus efficiently eliminated.

In human, NK cells are important in tumor immune surveillance as was demonstrated by the observation that low NK numbers lead to an increased risk of cancer. In leukemia patients, more evidence for a role of NK cells in tumor immune surveillance was provided by the observation of reduced receptors on NK cells in leukemia. Furthermore, leukemia patients that received an allogeneic stem cell transplantation that lack HLA class I ligands for donor inhibitory killer receptors had better clinical outcome.

The adaptive system predicts that tumor antigens in the context of MHC class I and II molecules are presented to T lymphocytes leading to a tumor specific reaction. To mount an adaptive immune response, two signals are mandatory. The first signal is provided by the presentation of an antigen in the groove of an MHC molecule to the T cell receptor (TCR). The second signal is provided by the ligation of co-stimulatory molecules (the most important being CD28 on T cells interacting with CD80 and CD86 on the antigen presenting cell (APC)). Antigen presentation in the absence of co-stimulation will induce anergy in stead of activation. Endogenous tumor antigens are presented by MHC class I molecules to cytotoxic CD8+ T cells. Also, tumor antigens can be presented by professional APCs in the groove of MHC class II molecules to naïve CD4+ T cells. APC will conduct naïve CD4 T cells to differentiate into Thelper1 (Th1), Thelper2 cells (Th2), Thelper17 (Th17) or regulatory T cells (Treg). Th1 cells are able to directly activate cytotoxic T cells but are also capable of directly killing tumor cells themselves. Th2 cells activate B cells that might produce antibodies directed against tumor cells. Tregs have immune suppressing activity and can counteract immune surveillance in solid tumors, acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). The role of Th17 cells in tumor immunology is not yet completely understood. Effective immune surveillance executed by the adaptive immune system is believed to rely on the generation of specific CD8+ cytotoxic T cell responses against tumor cells. For the induction of an effective anti-leukemic T cell response, however, CD4+ T cells are indispensable, providing help to cytotoxic T cell lymphocytes upon activation by APCs.

After successful recognition of the tumor cell by innate and adaptive effector cells, induction of apoptosis will follow. Despite recognizing target cells in very different ways, cytotoxic T lymphocytes and NK cells both utilise a pore-forming protein, perforin, and a battery of serine proteases (granzymes) that activate caspases, leading to apoptosis. The apoptotic program can also be activated...
after binding of TRAIL or Fas-ligand, expressed on the cell surface of the effector cell, to one of its cognate receptors. This will lead to the formation of a Death-Inducing Signaling Complex (DISC) consisting of the receptor, the FAS associated death domain adaptor protein (FADD) and caspase 8^{32;33}. Activated caspase 8 can directly activate caspase 3 leading to apoptosis and also, by cleaving Bid, activate the mitochondria-intrinsic pathway^{34}. As well as killing cells directly, cytotoxic T cells can act indirectly via cytokines. IFNγ and tumor necrosis factor alpha and beta (TNFα and TNFβ) are examples of this.

**Immune editing and subversion**

Evidence for the process of editing, subversion, and finally selection of less immunogenic tumor cells was provided by several studies in which tumors growing in immune-competent and in immune-incompetent mice were transplanted. It was demonstrated that tumors growing in immune-competent mice became ultimately less immunogenic compared to equivalent tumors growing in immune-incompetent mice^{35}. Tumor cells have developed numerous strategies to change their phenotype and mislead the immune system that can be categorized roughly in three groups.

1. Tumor cells can change their cell surface phenotype to prohibit recognition and binding by immune effector cells. The most well-known example of this strategy is the downregulation of MHC class I molecules (or molecules involved in antigen processing by MHC class I molecules) resulting in defective binding to the TCR of cytotoxic CD8+ T cells^{36;37}. However, as discussed above, downregulation of MHC class I renders cells susceptible for NK cell mediated killing. Consequently, tumor cells have also developed mechanisms to escape from NK surveillance: for example upregulation of the NK inhibitory HLA-G^{38}. Other strategies are the down-modulation of co-stimulatory molecules or upregulation of pro-apoptotic molecules like FAS-ligand on their cell surface to induce apoptosis of effector cells^{39}.

2. Another strategy is not to prohibit effective recognition but to impede the pro-apoptotic signals after effective recognition and binding of immune cells. An example of this evasion of immune mediated killing is to downregulate pro-apoptotic death receptors or to upregulate anti-apoptotic or decoy receptors (for example TRAIL-R3). Over-expression of anti-apoptotic proteins (like XIAP (X-linked inhibitor of apoptosis)^{40;41} or molecules that block the granzyme B/perforin pathway like (pI-9)^{42} are other examples.

3. The third way of subversion of the immune system is to produce immune suppressive factors that inhibit T cell function and lead to a local state of tolerance and anergy. Examples of this strategy are the production of transforming growth factor beta (TGF-β)^{43} or indoleamine 2,3-dioxygenase (IDO)^{44}. IDO degrades the essential amino acid tryptophan into kynurenine.
High levels of IDO result in immune suppression because T cells undergo cell cycle arrest in G1 phase at low tryptophan levels.\textsuperscript{45}

**Immune escape**

As a result of above mentioned strategies, non-immunogenic tumor cells have been able to grow out. These cells can produce immune suppressive enzymes, cytokines and may induce the development of regulatory T cells. All of these will ultimately lead to immune anergy, escape from immune surveillance and tumor progression. In the case of AML, the leukemic burden will inevitably be treated with chemotherapy (and eventually stem cell transplantation). Remaining minimal residual disease (MRD) cells which also escaped (were resistant to) chemotherapy and cause relapse, could ideally be represented to the immune system to provoke an effective immune response in second instance. A schematic overview of this process is given in Figure 1. Understanding the factors that result in immune subversion and escape establish the principles of developing effective immune therapy and forms the subject of this thesis.

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**Figure 1.** Schematic overview of successful tumor immune surveillance (left part), immune editing and escape leading to tumor progression and minimal residual disease (right part). The middle part (in gray) indicates the position and scope of this thesis.
Scope of the thesis

In this thesis, we have taken an immunological point of view to investigate the development and relapse of leukemic malignancies, mainly in patients with AML. Tumor immunology in the pathogenesis of hematological malignancies deserves special attention considering the difference with solid tumors. For example, regarding the intrinsic factors, metastasizing is common. Furthermore, developing hematopoietic tumor cells are from the beginning and throughout their lifecycle in close contact with mature cells of the immune system. This prolonged contact facilitates probably more effectively the shape of leukemic cells to non-immunogenic tumor cells when compared to solid tumor cells that develop in the relative absence of immune-competent cells. In the case of leukemic cells, the originating defect in the hematopoietic stem cell could also affect the development of mature immune cells, leading to dysfunctioning T and NK cells. These potential defects make developing immune therapy for leukemic patients an even more striking challenge. Although about 80% of AML patients achieve a first complete remission after intensive induction chemotherapy, relapses still occur in the majority of these cases\textsuperscript{46}. In the scope of this thesis, leukemic blasts, which escaped from immune surveillance in first instance, have acquired a non-immunogenic phenotype. They also survived induction treatment, and have created a situation of MRD. From the situation of MRD, 50% of patients develop a relapse of the disease\textsuperscript{46}. The ultimate goal of research performed in this thesis is to provide tools for the development of effective immunotherapy in the phase of minimal residual disease. In other words, we will try to restore contact between the immune system and the tumor cell that will lead to eradication of the residual tumor cells. To this end, non-immunogenic tumor cells will have to be turned into immunogenic cells, the apoptotic machinery has to be intact, and the immune cells will have to be freed from immune suppressive factors. The first prerequisite for development of a successful interventional therapeutic strategy for leukemic patients is to understand the tactics leukemic cells employ to play their hide and seek play with the immune system. To this end we investigated how immune cells interact with leukemic cells. In part A we evaluate the role of the adaptive immune system and investigate the recognition of leukemic cells by CD4\textsuperscript{+} T cells, which are deemed indispensable for generating an effective and long-lasting immune response\textsuperscript{31}. During HLA class II synthesis, class II $\alpha$ and $\beta$ chains dimerize in the endoplasmic reticulum (ER) and interact with the Invariant Chain (Ii) to form a nonameric complex. This interaction with Ii contributes to proper folding and prevents premature peptide loading of the HLA class II molecule\textsuperscript{47;48}. Furthermore, Ii plays an important role in targeting the HLA class II molecule from the ER.
into the endosomal/lysosomal pathway until the MHC class II antigen loading compartments (MIICs) are reached. In these compartments, II is proteolytically cleaved and a small peptide remnant, called the class II-associated invariant chain peptide (CLIP), remains associated with the antigen-binding groove. For the binding of exogenously derived antigenic peptides, which are processed in the endosomal/lysosomal pathway, CLIP has to be released from the antigen-binding groove by a specialized HLA-like chaperone, termed HLA-DM. In B cells, another molecule residing in MIICs, HLA-DO, is able to down-regulate the catalytic function of HLA-DM, thereby limiting exogenous antigen presentation on APCs.

In tumors lacking HLA class II expression, tumor-specific CD4+ T cell activation can only be induced by professional APCs that are loaded with tumor antigens. Studies in mice have demonstrated that tumor cells transfected with HLA class II genes are able to present tumor antigens on the cell surface and can induce strong CD4+ T cell responses. Interestingly, these and other studies further revealed that II expression in HLA class II-transfected tumor cells is adversely involved in activating tumor-reactive CD4+ T cells. This assumes that the absence of II in HLA class II-expressing tumor cells contributes to efficient HLA class II presentation of endogenous, i.e. tumor-associated antigens, leading to activation of tumor-specific CD4+ T cells.

In AML, myeloid leukemic blasts have an intrinsic property to express both HLA class II and co-stimulatory molecules on their cell surface. In this thesis, we analyzed MHC class II antigen presentation capacity (as read out by high CLIP expression) on AML blasts. We made correlations with clinical parameters (chapter III) and performed functional experiments in which II and CLIP were down modulated by siRNA transfection (chapter IV). The expression levels of the key regulators of MHC class II antigen presentation (HLA-DM and HLA-DO) were also investigated in myeloid leukemic cell lines and primary AML blasts. In chapter V, MHC class II, CLIP, HLA-DO, and HLA-DM expression on malignant cells of the lymphoid origin (chronic lymphocytic leukemia) is described and correlated to immune activation status.

In part B we evaluate the role of both adaptive and innate immune responses in patients with AML and in patients with myelodysplastic syndrome (MDS). If immune surveillance plays a role in the eradication of tumors, patients with premalignant diseases would have an activated immune status (for which evidence can be found in the case of patients with monoclonal gammopathy and pancreas carcinoma). To this end, we evaluated in chapter VI the role of immune surveillance in patients with MDS, which can be regarded as the premalignant phase of AML. Myelodysplastic syndromes are defined as a group of myeloid neoplasms characterized by morphologic dysplasia in one or more of the hematopoietic cell lineages. Increased proliferation of hematopoietic precursor...
cells in the bone marrow is counterbalanced by increased intramedullary apoptosis. This phenomenon is considered to underlie ineffective hematopoiesis which results in refractory peripheral cytopenia\textsuperscript{62}. MDS patients have a variable risk of transformation to AML. As a consequence of the high apoptotic load of the dysplastic and normal hematopoietic precursor cells, tumor-antigens and auto-antigens might be presented to the immune system and evoke an adaptive immune response. Consequently, activated T cells and clonal T cell expansions are found in the majority of MDS patients\textsuperscript{63-67}. However, the exact functional significance of these T cells remains unclear\textsuperscript{68}. On the one hand, the immune response could be directed to the dysplastic pre-malignant precursor cells and represent immune surveillance\textsuperscript{69}. On the other hand, as a consequence of breaking peripheral tolerance, undesired autoimmune reactivity against normal hematopoietic precursor cells could prevail. T cells as part of the adaptive immune system have been thought to play the dominant role in immune surveillance of MDS patients. Innate immune responses against tumor cells have been reported to play a role in immune surveillance in AML, as was demonstrated by a correlation between decreased NK cell activity and poor prognosis\textsuperscript{20,70}. In MDS, two groups have demonstrated normal frequencies of NK cells. Although different expression levels of NK activating receptors were reported, both groups reported reduced NK cell function\textsuperscript{71,72}. These data point to a primary role for the adaptive immune system in the pathogenesis of MDS, but many questions remain. Only 30% of patients respond to “classical” immune-suppressive therapy\textsuperscript{73,74}. Understanding the role of the immune system is crucial to identify patients that will benefit from immune-suppressing therapies as well as to correctly monitor patients that will be treated with immune-modulatory agents which are now rapidly introduced in treatment of MDS patients.

Furthermore, we evaluated two other strategies of AML cells to subvert the immune system. In chapter VII we studied resistance of leukemic cells to TRAIL induced apoptosis. Lymphocytes, NK cells, dendritic cells and monocytes are able to upregulate membrane bound TRAIL (mTRAIL) and to secrete a soluble form of TRAIL (sTRAIL) after stimulation with interferons or IL-2\textsuperscript{75-78}. In human, four membrane bound receptors for mTRAIL and sTRAIL have been identified: two of them, TRAIL-R1 and TRAIL-R2, contain a functional death domain and are capable of initiating the apoptotic cascade, while two others, TRAIL-R3 and TRAIL-R4, lack a functional death domain and function as decoy (antagonistic) receptors.

Several important functions for TRAIL induced apoptosis have been reported. First, TRAIL mediated cytotoxicity plays an important role in innate and adaptive immune responses\textsuperscript{79}. Second, TRAIL exerts a regulatory function on erythroid and myeloid maturation in normal hematopoiesis\textsuperscript{80-83}. Also senescent neutrophils are eliminated by TRAIL induced apoptosis upon their return to the bone marrow\textsuperscript{84}. Finally and most intensively studied, TRAIL has an important function
in tumor immune surveillance. TRAIL deficient mice are more susceptible to
tumor development and metastasis. One of the explanations for the tumor
selective activity of TRAIL has been the observation that normal cells mostly
express the decoy receptors TRAIL-R3 and TRAIL-R4, while many tumor cells
express TRAIL-R1 and TRAIL-R2 (reviewed in). In chapter VII, TRAIL receptor
expression is correlated to clinical outcome and a therapeutic strategy to restore
TRAIL sensitivity is explored.

Finally, in chapter VIII, the role of the immune suppressing enzyme IDO
(indoleamine 2,3 dioxygenase) in AML cells is explored. Successful immunotherapy
requires not only the development of effector T cells, but also the break-through
of the local state of immune tolerance that tumors can create. Besides several
cellular and soluble factors (like the presence of regulatory T cells and TGF-
ß), over-expression of the enzyme IDO can induce tolerance. IDO degrades
the essential amino acid tryptophan into kynurenine. High levels of IDO result
in immune suppression because T cells undergo cell cycle arrest in G1 phase at
low tryptophan levels. Moreover, kynurenine and its derivates are directly toxic
for T cells. Biologically relevant IDO to limit T cell activation is expressed by
IFNγ stimulated APC, in lower intestinal epithelial cells in which non-pathogenic
bacteria are frequently present, and in trophoblast cells in which it protects the
foetus from attack by maternal cytotoxic T cells. Tumor induced over-expression
of IDO causes immune-suppression at two levels. First, inhibition of effective
T cell priming by APC-derived IDO has been demonstrated in tumor-draining
lymph nodes. Second, the effector phase of an anti-tumor immune response
is hampered because many human solid tumors itself express IDO. With
regard to hematological malignancies, Curti et al reported active IDO protein
in 52% of AML samples. They also clearly demonstrated that IDO expressed
by human myeloid leukemic cells induces the expansion of CD4+CD25+FOXP3+
regulatory T cells in mice. These data presume an important role for IDO in
immune escape. However, negative influence of high IDO expression on overall
survival of patients has only been demonstrated for endometrial and colorectal
cancer. We present data about the expression of INDO (the gene encoding
for IDO) in AML samples and correlate these to clinical outcome.
Reference List


