General discussion and future perspectives

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Introduction

In 1811 William Cullen described a case of ‘splenitis acutus’ in which the serum of the blood had the appearance of milk; with our current knowledge Cullen is most likely the first to describe a case of chronic leukemia. In 1827 a more detailed case report on a 63 year old patient, Mr Vernis, was published by Alfred Velpeau. Mr Vernis’s illness started at the age of 54 and was followed by a stable phase for three years. This phase was followed by a period of recurrent fevers and difficulty urinating. Eventually, the patient was admitted to hospital at the age of 63 with tumors in the abdomen and died the next morning. An autopsy revealed an enlarged liver and spleen and pus-filled blood. Then, Alfred Donné discriminated in his morphological atlas (published in 1845) between accumulation of pus in the blood as seen in infections or abscesses and of so called “mucous globules” (leucocytes) as he found in a case of a 44-year old woman with splenomegaly. John Bennett stated in 1845 that in case of leucocytosis this should be considered as a primary systemic blood disorder. In 1847 Rudolf Virchow was the first pathologist to describe this clinical and pathological condition as leukemia based on the Greek words leukos (λευκός), and aima (αίμα), meaning white and blood respectively. With further development of microscopy of blood smears Virchow was able to differentiate between splenic and lymphatic leukemia in 1856; it was not until 1869, when Neumann discovered that the origin of leukaemia should be connected to the bone marrow. Further development of stained blood smears designated the beginning of a new era in which more details of leukemia were unravelled but yet many more complicated questions raised [1].

Figure 1. Anatomy lessons of Dr Velpeau. 1864, by Francois Nicolas Augustin Teyen-Perrin.
Models for the development of leukemia

Over the last decades, more and more is revealed about the development of cancer. Simplified models have been proposed, however, none of these are simple. For instance, massive exposure to radioactive substances will lead to breaks in double stranded DNA and thereby translation into proteins may stop which finally leads to death of hematopoietic stem cells: acute radiation syndrome. When one is exposed to lower amounts of radiation, this will lead to multiple DNA breaks which can be repaired by the cell itself. When this mechanism fails, the improperly repaired DNA might encode for proteins which promote unlimited growth of hematopoietic stem cells and in this case in months to years leukemia might develop. Especially mutations in DNA repair mechanisms or other tumor suppressor genes are well described. For instance, the P53 gene plays a decisive role in DNA repair and it can induce cell cycle arrest to provide time for DNA repair and when repair is not possible P53 can induce apoptosis. Mutation in P53 genes and abolishment of its function can promote development of cancer [2]. However, no model can yet explain why one hematopoietic progenitor cell becomes malignant and another progenitor cell will not. Moreover, the development of models answering these questions is complicated by the heterogeneity of leukemic cells between patients and even within one patient. Nevertheless, different models are developed trying to explain the heterogeneity of a tumor: the stochastic model points out that tumors are inherently biologically homogenous and heterogeneity is caused by random intrinsic or extrinsic influences that alter the behavior of individual cells in the tumor. In contrast, the deterministic model states that heterogeneity of tumors is caused by known influences and relationships between other cells and the environment, a role for random phenomena is excluded [3]. In some cases, a pre-leukemic phase of ineffective production and dysplasia of the myeloid cell lineage, the myelodysplastic syndrome (MDS), might precede evolution to AML. In these cases the steps to malignant evolution into AML can be researched more thoroughly: Walter et al. proposed a model for the development of secondary AML from MDS based on single nucleotide variant analysis performed at various time points during evolution of MDS to AML (summarized in figure 2)[4]. All of these hypotheses have provided the basis for better understanding cancer and consequently...

Figure 2. A model explaining clonal evolution (adapted from Walter et al. [4]). Based on Single nucleotide variant analysis of MDS (green cells) and secondary AML (purple) the following model was proposed for the evolution of MDS into AML. First (1) a single clone (green) is present. Cells in clone 2 (red) are derived from a single cell from clone 1 since newly acquired variants are found in addition to the same single nucleotide variants found in nearly all AML cells. Finally, AML has developed showing various subclones evolved through serial (2,3) acquisition of single nucleotide variants.
added to the development of new therapeutics and as a result, survival rates in leukemia have increased over the last decades. Nowadays, after induction and consolidation regimens complete remission can be established in 70-80% of adults with acute myeloid leukemia (AML) and even better results for acute lymphoid leukemia (ALL). Unfortunately, 5 year survival rates are significantly lower, for AML this is 30-40% and for ALL 40-50%.

The classification of acute (myeloid) leukemia

Despite heterogeneity as described above, in clinical studies AML patients have been regarded as one homogenous group, randomized in two cohorts: one for the standard and one for the new (chemo)therapeutics. With our current knowledge, treatment regimens may differ for patients with a (very) good and poor risk profile (see www.hovon.nl for Dutch treatment guidelines). These risk stratifications are primarily based on molecular and genetic aberrancies. Thus, as a start, there should be consensus on how to classify acute leukemias in order to provide a standardized platform for testing different treatment options for distinct subgroups of AML. A struggle on one hand is the heterogeneity of acute leukemias and potentially numerous subgroups and on the other hand the need for large (homogenous) groups of patients for testing new (chemo)therapeutic regimens.

In order to have a worldwide consensus on classification the WHO2008 provides guidelines for diagnosing different subgroups based on a combination of clinical, morphologic, and immunophenotypic features, and results obtained by molecular analysis and cytogenetics. Differentiation between AML and acute lymphoid leukemia (ALL) is important since ALL necessitates a different therapeutic regimen than leukemias from myeloid origin; for example, a longer maintenance treatment and intrathecal prophylaxis is needed for lymphoid leukemias.

In PART I of this thesis we evaluated the role of the WHO2008 guidelines in differentiating classification of myeloid and lymphoid leukemias by flow cytometry. In chapter 3 we describe two case reports highlighting the importance of immunophenotyping. The first report is a lymphoid leukemia at diagnosis which relapses as an immunophenotypic myeloid leukemia with the same complex cytogenetic aberrancies as at diagnosis. This subclone with a myeloid phenotype was already present at diagnosis and after chemotherapy following the ALL protocol. The other acute leukemia case describes a paradoxical appearance: morphology revealed a clear monocytoid picture with prominent vacuolization and erythrophagocytosis, whereas flow cytometry revealed a clear lymphoid phenotype. Besides the lymphoid markers, also MPO was found positive, thus classifying this case as a mixed phenotype acute leukaemia following the WHO2008 guidelines. The patient was treated with an AML chemotherapy regimen combined with intrathecal methotrexate prophylaxis, resulting in complete remission. Unfortunately the patient experienced a relapse (with the same phenotype) after 11 months, and further chemotherapy was not given. In about 2-4% of acute leukemias flow cytometry cannot unravel whether an acute leukemia should be assigned to the myeloid or lymphoid lineage. When expression of both myeloid and lymphoid markers is found, the acute leukemias are classified as mixed phenotype acute leukemias (MPAL), it is unclear whether MPAL benefit from an ALL or AML based treatment protocol. Moreover, acute leukemias with both lymphoid and myeloid
features have a poor prognosis and therefore may benefit from an intensified treatment scheme. International guidelines implementing these intensified schemes are lacking. For comparison of these different chemotherapy schemes a uniform classification has to be used. However, the last decades various guidelines have been used for classification of acute leukemias of ambiguous lineage. In chapter 4 we discussed the implications of the various guidelines and compared the latest WHO2008 guidelines with the former WHO2001 guidelines in classification of MPAL. Noteworthy, according to the WHO2001 criteria 5.8% of all acute leukemias referred to our institute were classified as acute leukemias with both lymphoid and myeloid features (biphenotypic acute leukemia (WHO2001)), whereas according to the WHO2008 guidelines only 1.3% would have been classified as MPAL. In addition, clearly defined cut offs for positivity and negativity of certain immunophenotypic diagnostic markers are lacking in WHO2008; e.g. no cut off for cytoplasmic myeloperoxidase (cMPO), the hallmark of the myeloid lineage is defined. In chapter 5, we demonstrated that a 10% cut off is a secure lower limit for MPO expression by flow cytometry and can be used independently from the cytomorphology based Sudan Black analysis. Compared with a regular cut off of 20% (according to the former WHO2001 guidelines), this proposed cut off of 10% for cMPO expression would have changed the diagnosis into MPAL in only two cases in our cohort (1%). Nowadays, treatment schemes for MPAL are based on a lymphoid or myeloid based treatment scheme, the latter often combined with intrathecal methotrexate prophylaxis. Accordingly, it is necessary to distinguish between a myeloid or lymphoid lineage predominance within an acute leukemia with both characteristics. Classification of MPAL might be complemented by the use of other diagnostic tools, such as gene or microRNA expression profiling. In chapter 6 we demonstrated by microRNA arrays that MPAL did not segregate as a separate entity but showed microRNA expression profiles similar to that of either AML, B-ALL or T-ALL. This implies that MPAL might not be a unique clinical entity but can be traced back to their original genotypic lineage using microRNA expression. We hypothesize that microRNA classification of those leukemias with both lymphoid and myeloid characteristics could provide a more accurate classification as compared to immunophenotyping and is instrumental for therapy decision making. However, prospective studies are needed for implementation of microRNA arrays in clinical practice.

In addition to microRNA arrays, the development of other mass data analysis on a gene or protein level (such as gene arrays, proteomics and mass cytometry [5]) will help to define more accurate signatures for acute leukemias and leukemic cells and thus more accurate definitions of subgroups of acute leukemias. In the near future these diagnostic tools might help defining (sub)groups for comparing different therapeutic schedules. Ideally, a personalized leukemic signature might lead to a personalized treatment: for instance a personalized immunotherapy which is discussed in PART III.

The development of AML: immunesurveillance

Most models describing the role of the immune system in cancer are deduced from the innate and adaptive immune response to foreign micro-organisms. One should be careful to extrapolate these models to acute leukemia. For instance, the process of a pre-malignant cell evolving into leukemia might take years such as is the case for MDS, whereas micro-
organisms can cause fulminant infections within days. Furthermore, in contrast to micro-organisms, leukemic cells are derived from previous non-malignant cells and thus express more self than non-self-antigens. Nonetheless, these models provide insights in the role of the immune system in the development of cancer and thus might provide a tool for the development of therapies. One interesting model trying to explain the development of leukemia is discussed in this thesis and is based on escape of the immune system. In this model the immune system is depicted as a balance (figure 3). A balanced immune system reacts in a balanced manner to micro-organisms and malignant cells (figure 3b). An overacting immune system (figure 3a) results in hyper inflammation and even worse, an immune response against normal cells and subsequently auto-immune diseases. In contrast, in case of hypo-inflammation (figure 3c), the immune system leaves (pre)leukemic cells untouched resulting in outgrowth of leukemia. In other words, in order for leukemia to develop the leukemic cells must acquire escape mechanisms to prevent recognition by the immune system, this is researched in PART II of this thesis. However, this model can not be generally applied: for instance, chronic myeloid leukemia (CML) cells are able to activate BCR-ABL-specific T cells, however, these T cells were not able to proliferate [6]. Furthermore, it is evidenced that persistent antigen exposure can result in loss of high affinity leukemia reactive T cells or T cell tolerance [7-10]. Besides, many other immunosuppressive mechanisms are described involving the tumor environment such as regulatory and suppressor T cells and molecules expressed by the tumor cell itself such as indoleamine 2,3-dioxygenase (IDO) and Class II associated invariant chain peptide (CLIP) [11;12]. In PART II we have investigated the role of class II associated Invariant Chain peptide (CLIP) in antigen presentation via HLA class II by leukemic cells as a possible immune escape mechanism. Previously, we have shown that patients with a high CLIP expression on leukemic cells have a worse prognosis [12]. In this thesis we showed that CLIP expression on minimal residual disease can predict relapse more accurately (Chapter 8). The model explaining this difference in survival assumes that CLIP is preventing presentation of leukemia associated antigen (LAA) derived peptides by HLA-class II. However, CLIP expression is regulated by HLA-DM and HLA-DO; as such, CLIP expression might be the result of alterations in other pathways and molecules. CLIP expression implies that exogenous proteins are not efficiently presented by the leukemic cell onto HLA class II molecules. This assumption is substantiated by the finding that leukemic cells positive for CLIP have reduced CD4+ T cell activating capacity compared with the CLIP negative cells.

Figure 3. A simple (A) and complex (D) model explaining immunologic processes. In (A) the immune system is represented as a balance. In A an over-reactive immune system will result in auto-immune diseases and an unresponsive (C) immune system will result in the development of cancer. When the immune system is balanced (B) no auto-immune diseases will develop and the immune system is capable of eliminating cancer. I
within the same patients in an autologous setting (Chapter 9). These results have led to
the assumption that analysis of CLIP can be used to predict which patient might be likely to
experience a relapse. High CLIP expression results in a poor prognosis and warrants more
intensive chemotherapy, or more preferably therapy directly targeting CLIP. The latter may
entail potentiation of the antigen processing machinery, such as the proteasome and TAP.
For instance, clinically applicable HDAC inhibitors enhance the expression of TAP subunits
in tumor cells. By incubation of leukemic cells with HDAC inhibitors in vitro we found strong
down modulation of CLIP (unpublished data). Another option might be to directly inhibit
CLIP expression via modulation of HLA-DM/DO, for instance using PKC inhibitors [13]: in
activated B cells PKC inhibition affects DO expression potentially leading to a decline in CLIP.
In chapter 15 we investigate the role of R848 on monocyte derived dendritic cells (MoDC)
and show down-modulation of CLIP. The role of TLR-ligands in affecting CLIP expression
on leukemic cells should be further investigated; preliminary experiments showed that
by incubation of leukemic cells with TLR-ligands CLIP was down regulated. However, due
to presence of CLIP on non-malignant cells, such as B-cells one should be very careful in
application of CLIP modulation in clinical practice in order to prevent auto-immunity.

Models for development of immunotherapeutic strategies

The immune-escape model provides a model for the development of cancer but can also
provide a starting point for new treatment modalities such as immunotherapy (vaccination).
After initial chemotherapy the bulk of leukemic cells are lysed, this is followed by
consolidation therapy to attack remaining residual cells. In addition to consolidation therapy,
immunotherapy can add to the recognition and lysis of residual leukemic cells and thereby
might prevent relapse of AML. Immunotherapy aims at the induction of leukemic antigen
specific T cells. In chapter 11 we show that PRAME-specific T cells efficiently recognize
and lyse leukemic cells and thus are an attractive tool for development of additional
immunotherapeutic strategies, such as adoptive T cell transfer or active immunotherapy
aiming at the induction of PRAME or other LAA-specific T cells
The critical role of dendritic cells (DC) in induction, regulation and maintenance of primary
immune responses, including specific antitumor responses, has been demonstrated. This has
led to development of DC-based vaccination strategies as a way to actively induce antitumor
immunity. Thus far, results are promising: T cell responses have been elicited by DC vaccines
and DC vaccines have proved to have only few side effects [14;15]. Unfortunately, major
clinical responses have only been reported in a minority of cancer patients. Partly this can
be explained by the fact that DC-based vaccines have been administered to cancer patients
who obtained a second complete remission (CR) after relapse and thus have a very poor
prognosis; these results highlight the need for improvement of DC-based immunotherapeutic
strategies. For the latter, lessons can be learnt from stem cell transplantations (SCT): whereas
the patients’ immune system fails to control leukemic cells, donor immune cells can induce
long term CR due to activation of donor T cells. These donor-derived T cells recognize either
alloantigens that are expressed on normal and malignant cells or leukemia-specific antigens.
However, SCT is not suitable for all patients, for instance those patient with high morbidity
or older patients, and has a high treatment-related mortality and morbidity (graft versus host disease). Passive immunotherapy might be effective in the short term; it does not offer long term immunity. In contrast, active DC-based immunotherapy aims at the induction of the LAA-specific T cells including memory T cells. The leukemic cell contains already known and not yet discovered LAA and thus provides an attractive source for loading onto DC and for development of personalized treatment regimens. Another option is to use DC cultured from leukemic cells: by culturing leukemic cells with a cytokine cocktail consisting of GM-CSF and IL-4 differentiation into leukemic cell-derived DC is potentiated; this technique is exploited in chapter 12. In favor of this concept is that leukemic cells and leukemic cell-derived DC processes and presents the same leukemic antigens by MHC-molecules. However, the leukemic cell-derived DC might harbor immune suppressive features and the antigen presenting machinery is less efficient as compared to normal non-leukemia derived DC. [16]. Another option is the use of MoDC loaded with leukemia-associated antigen sources. There are various ways to supply leukemic cells to the dendritic cells. For instance by repetitive freeze (liquid nitrogen)/ thaw (42°C) cycles cellular integrity is lost and intracellular proteins and peptides can be provided to the DC for uptake and further processing. In chapter 13 we show that CML lysate-loaded DC are able to activate CML-specific T cells. Another option is the induction of apoptosis by for instance, heat-shock, UV- irradiation or chemotherapeutics to render leukemic cells suitable for loading onto MoDC. Beside the numerous antigen sources (e.g. lysate, blebs, RNA, peptides) even more options for adjuvants potentially enhancing the loading efficacy are becoming known. In chapter 14 we have compared different DC-loading strategies for optimal immune responses. To shorten the list of numerous DC loading strategies to a researchable amount we focused on whole cell products: lysate and apoptotic cells. In innate immunity toll like receptors (TLR) are necessary to induce an optimal innate immune response and help the adaptive immune response. In contrast to lysate, by loading apoptotic cells in presence of TLR ligand more antigen is taken up by the DC (chapter 14). The addition of TLR ligands during loading of MoDC enhances the uptake of leukemic cell lysate and apoptotic cells. However, the sequential use of R848 and a conventional cytokine cocktail is counter indicated due to its adverse effects on MoDC maturation.

During apoptosis of non-malignant cells highly immunogenic blebs are shed; the remaining apoptotic body is considered less immunogenic. We explored the immunogenicity of apoptotic blebs in leukemic cells in chapter 15 and show that bleb-loaded DC are more efficient in T cell activation than the apoptotic cell remnants. Further systematic studies comparing leukemic cell derived DC and MoDC loaded with leukemic cell-derived antigens sources are needed to evaluate the best DC source for inducing LAA specific T cells.

Future directions for development of immunotherapy

Only a few studies are available comparing different DC loading strategies [17-20]. These comparative studies revealed that different AML patients might benefit from different vaccine preparations. Furthermore, comparison of various studies with each other is complicated by different MoDC culturing and subsequent loading procedures. In this thesis, we show that LAA-specific T cells can efficiently recognize and lyse leukemic cells. Furthermore, for the development of active specific immunotherapy, DC-based
vaccines offer an interesting option. We show that leukemic cells can be differentiated into DC, whether or not after preceding expansion of leukemic cells. Furthermore, loading of MoDC loaded with blebs is favorable over loading with apoptotic cell remnants. And when loading the DC, one should be careful with the use of adjuvants such as TLR-L. These recommendations, which we provide in this thesis, offer a starting point for further research: these studies should focus on comparison of variations in DC culturing and loading possibilities. For example, AML-derived DC have not yet been compared side-by-side with MoDC loaded with whole cell derived antigens.

Only a few LAA are known and these are not generally expressed in AML and thus hamper a LAA-specific in vitro read-out system [21]. In these cases results obtained from research using CML-cell loaded MoDC might be helpful: BCR-ABL is generally expressed in CML and phenotype and genotype are more homogenous compared with AML.

When taking the stem cell model into account another challenge is faced in DC loading strategies: DC primed LAA specific T cells should ideally target leukemic cells and leukemia initiating stem cells. Therefore, in vitro research should focus on the development of leukemic stem cell loading strategies onto DC, for instance by loading of amplified RNA isolated from the leukemic stem cell.

Summary

In summary, in PART I we described the struggle with on one hand the heterogeneity of acute leukemias and potentially numerous subgroups and on the other hand the need for large (homogenous) groups of patients for testing new therapeutic regimens. Immunophenotyping plays an important role in definition of various subgroups. In future, results obtained from e.g. gene or microRNA array or mass cytometry might add to deciphering the genetic and protein signature of a leukemic cell and more accurate definition of subgroups. In PART II we showed that leukemic cells exploit immune escape mechanisms such as expression of CLIP. These immune escape mechanisms provide a starting point for developing of immunotherapeutic interventions as described in PART III of this thesis. In this part we evaluated different DC-based loading strategies and made a start in finding the most optimal vaccine preparation and compare different adjuvants potentially enhancing DC-preparation and function. Diagnostic tools have to be developed that can direct personalized treatment schemes, such as immunotherapeutic vaccination strategies.

Several steps have to be taken for preparation of dendritic cell based vaccines: ongoing research is needed in order to achieve consensus about which antigen loading strategy for MoDC combined with administration of optimal adjuvants is most suitable. This includes optimization of LAA processing and presentation pathways in order to achieve the best-suited tailor-made vaccine preparation in each individual patient.

Reference List