Chapter IX

Summary, conclusions, discussion, and future perspectives

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Introduction

The moment that cancer is diagnosed, there is usually considerable tumor burden. As a result of tumor immune editing, non-immunogenic tumor cells have developed and the immune system has become anergic or tolerant. The primary goal of immunotherapy should be to overcome tolerance and to re-educate the immune system, at the moment the tumor burden is reduced. Several approaches have been tested, varying from administration of cytokines to vaccination procedures. Vaccination can be achieved either by immunizing patients (with highly immunogenic tumor cell based or dendritic cell based vaccines[1]), or by adoptive transfer of antigen-specific T cells[2]. Many studies have shown that the immune system is able to raise anti-tumor activity (reviewed by Rosenberg[3] and Finn[4]) although broad application of these vaccines is not yet established.
In the case of acute myeloid leukemia, about half of patients that achieve complete remission relapse within two years. Apparently, residual leukemic cells have survived immune surveillance and chemotherapy. The successful application of allogeneic stem cell transplantation and subsequently donor lymphocytes infusions has clearly demonstrated that leukemic cells can be recognized and eliminated by immune cells. For allogeneic settings as well as for autologous settings, fundamental knowledge about how leukemic cells are recognized and escape from immune surveillance will add significantly to the development of successful immunotherapy.

Chapter summaries

Chapter I starts with a short general introduction on tumor immune surveillance, immune editing, immune subversion, and immune escape. The scope of the thesis is presented, which comprehends to reveal several aspects of the interaction of leukemic cells with the immune system. To develop successful therapeutic strategies, understanding of the tactics leukemic cells employ to subvert the immune system is fundamental.

In **chapter II**, the particular role of MHC class II molecules in tumor immunology is reviewed. MHC class II molecules activate $CD4^+$ T cells which are the central orchestrating cells of an immune response. The MHC class II antigen presentation pathway and the expression of MHC class II molecules on tumor cells related to clinical outcome is discussed.

In **chapter III**, we report that CLIP was expressed on the cell surface of AML blasts. Patients with HLA-DR^+/$CLIP^-$ blasts had a significant longer disease-free survival than patients with HLA-DR^+/$CLIP^+$ blasts. We show that differences in

class II antigen presentation are associated with the clinical outcome of AML patients. HLA-DO, until now believed to be restricted to lymphoid cells[5], could be demonstrated at protein level as well as by reverse transcription-PCR. HLA-DO:HLA-DM ratio correlated to CLIP:HLA-DR ratio, suggesting that, unlike in other antigen-presenting cells of the nonlymphoid cell type, both HLA-DO and HLA-DM mediate regulation of CLIP expression in AML blasts. We hypothesize that HLA-DR^+/$CLIP^-$ AML blasts are able to present leukemia-specific antigens to $CD4^+$ T helper cells initiating an effective and long-lasting antitumor response resulting in a prolonged disease-free survival.

In **chapter IV**, data are presented that confirm the correlation between high CLIP expression on the cell surface of myeloid leukemic cells and poor clinical outcome in an expanded cohort of 207 AML patients. These additional data are in agreement with the hypothesis (as presented in chapter III) that leukemic blasts with both a low Ii and a low relative CLIP amount are able to activate leukemia-specific $CD4^+$ T cells, resulting in an effective anti-leukemic immune response *in vivo*.
Immunophenotypic screening of several human myeloid leukemic cell lines revealed large differences in relative CLIP amount. KG-1 and ME-1 blasts have a DR^+/Ii^+/$CLIP^-$ phenotype and THP-1 and Kasumi-1 have a HLA-DR^+/Ii^+/$CLIP^+$ phenotype. HLA-$DR^+CLIP^+Ii^+$ THP-1 and Kasumi-1 blasts could be completely down-modulated for Ii (and CLIP) expression by retroviral transduction of specific Ii siRNAs. Despite the similar immunophenotype of THP-1 and Kasumi-1 blasts, different effects of Ii down-modulation were noted on cell surface HLA-DR expression. In monocytic THP-1 blasts, we found that, in the absence of Ii, HLA-DR molecules were still expressed on the cell surface, where Kasumi-1/Ii-siRNA blasts exhibited reduced levels of cell surface HLA-DR expression. Despite differences in cell surface HLA-DR expression after Ii siRNA transduction of THP1 and Kasumi-1 cells, both transduced cell lines demonstrated a clear decrease in relative CLIP amount and were found to strongly enhance the activation of allogeneic $CD4^+$ cells in functional assays, indicating the enhanced immunogenicity of HLA-$DR^+CLIP^-Ii^-$ AML blasts.

In **chapter V,** we further investigated the MHC class II antigen presentation pathway in chronic lymphocytic leukemia (B-CLL). B-CLL cells showed a disturbed expression of HLA-DM and HLA-DO. The ratio of HLA-DM/HLA-DO was significantly increased in B-CLL cells when compared to normal B cells. The increased HLA-DM/HLA-DO balance altered the peptide repertoire, as it was related to a reduced expression of the self-peptide CLIP at the cell surface. CLIP expression on the cell surface of B-CLL cells correlated inversely with the percentage of activated T cells ($CD4^+$ and $CD8^+$). Thus, in B-CLL a relatively

increase in HLA-DM and a concomitant change in the MHC class II peptide repertoire of the malignant B cells is related to ongoing T cell activation.

If immune surveillance plays a role in tumor development, an activated immune system would be expected in patients with pre-malignant lesions. Myelodysplastic syndromes (MDS) can be regarded as the pre-malignant phase of AML[6]. Indeed, an activated immune system has been observed in MDS patients but its exact contribution to disease development and control is not fully clarified. On the one hand an activated and skewed T cell repertoire has been reported, but on the other hand, decreased NK cell function has been found[7;8]. Immune activation could reflect undesired autoimmune reactions against normal hematopoietic precursor cells as well as effective immune surveillance against dysplastic clones. In **chapter VI** we have investigated immune effector cells (lymphocyte subsets, lymphocyte activation markers, and NK cells) of low and intermediate risk MDS patients and compared them to those of age-matched healthy donors. Furthermore, we have analyzed the cytotoxic capacity of effector cells against autologous bone marrow hematopoietic precursor cells of MDS patients and healthy donors. In MDS patients, we have found an activated state of lymphocytes, determined by increased percentages of effector T cells with cytotoxic profile, more skewing of the T cell receptor Vbeta (TCR-Vß) repertoire, and decreased frequencies of regulatory T cells, when compared to healthy donors. The percentage of NK cells did not differ between MDS patients and healthy donors, but NK cells of MDS patients expressed increased levels of granzyme B. Finally, we have demonstrated non-MHC restricted autologous cytotoxicity up to 90% against aberrant hematopoietic precursor cells, presumably mediated by NK cells. These data provide evidence for active immune surveillance, mediated by both adaptive and innate immune responses, in the pathogenesis of MDS patients.

Chapter VII describes the possibility of myeloid leukemic cells to escape TRAIL mediated apoptosis. TRAIL can induce apoptosis in a broad range of human cancer cells. Four membrane-bound receptors for TRAIL have been identified: TRAIL-R1 and TRAIL-R2 contain a functional death domain. TRAIL-R3 and TRAIL-R4 lack a functional death domain and function as decoy receptors. Flow-cytometric determination of TRAIL receptors revealed that AML blasts expressed significantly more pro-apoptotic receptors compared to normal blasts. However, about 20% of AML patients had a high expression level of the anti-apoptotic TRAIL-R3, which was strongly correlated to a shortened overall survival. In multivariate analysis, R3 expression was a stronger prognostic factor for overall survival than age. Cell death induction of primary AML samples with sTRAIL/Apo2L was 14% (0-54%) and could be enhanced by down-modulation of TRAIL-R3, confirming its decoy function on AML blasts. Bypassing of TRAIL-R3

by treatment with antibodies directly targeting TRAIL-R2 resulted in high rates of induced cell death (mean 30%, 0-80%).
In conclusion, AML blasts do express pro-apoptotic TRAIL receptors. However, co-expression with the decoy receptor TRAIL-R3 results in significant shortened overall survival. AML blasts are sensitive to targeting the pro-apoptotic TRAIL-R2 receptor.

In **chapter VIII**, we have investigated a strategy that tumor cells can exploit to induce T cell suppression and tolerance. Indoleamine 2,3-dioxygenase (IDO) degrades the amino acid tryptophan which is essential for T cells. Tryptophan depletion causes T cell cycle arrest and solid tumors that express high levels of IDO can create immune suppression. Recently, blasts of patients with acute myeloid leukemia were shown to express indoleamine 2,3-dioxygenase. We determined *INDO* (encoding gene for IDO) mRNA expression in leukemic blasts of 286 patients with acute myeloid leukemia by gene-expression profiling. Results were validated by quantitative polymerase chain reaction analysis in blasts of an independent cohort of 71 patients. High *INDO* expression was correlated to significantly shortened overall and relapse-free survival. Correlation of *INDO* expression to relevant known prognostic factors and survival identified high *INDO* expression as a strong negative independent predicting variable for overall and relapse-free survival.

Discussion and future perspectives

We have found evidence for inefficient MHC class II antigen presentation as an immune escape mechanism of myeloid and lymphatic leukemic cells. In first instance, it was surprising to find an inverse correlation between better clinical outcome of AML patients and low CLIP expression at the cell surface of myeloid leukemic cells themselves. To understand this, two controversial assumptions had to be made. The first was that AML blasts have sufficient co-stimulatory molecules to activate $CD4^+$ T cells. The second, that endogenous tumor specific antigens are presented by MHC class II molecules. It was challenging to not immediately reject these findings that were in fact contradictory to what is described in immunology handbooks. We decided to conduct functional studies to prove the central hypothesis of part A: the positive correlation between effective MHC class II antigen presentation on tumor cells themselves and enhanced $CD4^+$ T cell activation. The functional data presented in chapter IV (mixed lymphocyte reactions with Ii-siRNA transduced myeloid leukemic cell lines), strongly supported the hypothesis. We have three more indications that support our hypothesis (experiments for this purpose are being expanded at the moment and for that reason not presented as a chapter in this thesis). First, in

the situation of minimal residual disease, myeloid leukemic blasts (identified by the expression of flow-cytometric aberrant markers) from patients that achieved prolonged complete remission had a significant lower relative CLIP expression than myeloid blasts of patients that relapsed soon after withdrawal[9]. Second, in patients with low-risk MDS, we have analyzed relative CLIP expression on the hematopoietic precursor cells before and after treatment with the growth factors erythropoietin and granulocyte-colony stimulating factor (G-CSF). Treatment with these growth factors is associated with less transfusion dependency and improved survival of myelodysplastic patients[10]. Besides the activated immune system found in these patients (chapter VI), we have also found a decrease in relative CLIP expression on the hematopoietic precursor cells after 1 year of treatment[11]. Presumably, better MHC class II antigen presentation is one of the factors that play a role in the improved survival of low risk MDS patients. Third, experiments conducted in our laboratory seem to confirm the ability of MHC class II molecules in leukemic myeloid cells to present endogenous antigens (submitted, M. van Luijn). In conclusion, impaired MHC class II antigen presentation is of prognostic significance in AML patients and enhanced MHC class II antigen presentation is correlated to immune activation in MDS and CLL patients.

The question is how these findings could be applied in the development of immunotherapy for leukemic patients. We propose that tumor cells themselves are able to elicit an immune response, however whether naïve or already activated tumor specific T cells are activated is not clarified. Likewise, tumor cells will maintain the risk of down-regulation of co-stimulatory molecules and consequently induce tolerance in stead of an effective immune response. We have therefore decided to incorporate the knowledge about efficient MHC class II tumor antigen presentation in the generation of dendritic cells (DC) for vaccination. In our laboratory, much work is already being done on the generation of DC vaccination for AML patients[1], which is recognized as an important investigational therapy[12;13]. Due to their unique antigen presenting capacity, immunosuppressive features of the leukemic blasts can be circumvented. DC's can be successfully cultured from leukemic blasts in 60-70% of patients and show functional potential in vivo. MHC class II transfected, Ii negative tumor cells present endogenous antigens and can act as successful vaccines that directly activate $CD4^+$ cells[14-16]. Alternatively, monocyte derived DC obtained at time of complete remission loaded with leukemia-specific antigens can be used as vaccine. Ii down-modulation could be used as an additional strategy in AML or monocyte derived DC to activate $CD4^+$ T cells specific for a broad range of leukemia-associated antigens. Several other interventions to augment MHC class II expression in tumor vaccines could be explored. As HLA-DO was marked as a factor to impair MHC class II presentation by hindering the function of HLA-DM in leukemic cells[17], downregulation of HLA-DO by antisense or siRNA techniques

could be explored. Some protein kinase C inhibitors downregulate DO in vitro[5], and use of them could provide another strategy to augment MHC class II tumor antigen presentation. Furthermore, the main cause of downregulation of MHC class II molecules in tumors is methylation of the promoter genes of CIITA. DNA methylation is believed to inhibit the expression of MHC class II molecules in haematopoietic tumor cell lines and AML patient samples by silencing its coactivator[18]. Hypomethylating agents are already intensively studied for use as anticancer therapy to reverse hypermethylation of tumor suppressor genes[19].

In part B other (than MHC class II related) interactions of myeloid blasts with the immune system are described. Immune-modulatory drugs are rapidly introduced in the treatment of MDS patients. To elucidate better the role of the innate and adaptive immune responses in the pathogenesis of myelodysplastic syndromes, a detailed investigational plan on the immunological parameters will now be incorporated in a phase II study in which low risk MDS patients will be treated with lenalidomide. Profound analyses of lymphocyte subsets and autologous cytotoxicity capacities will be performed and correlation to clinical outcome (progression to AML) will be observed prospectively.
Our results demonstrate that the *INDO* mRNA level is a strong independent predicting variable for the outcome of AML patients. Inhibition of IDO by orally available inhibitors like 1-methyl-tryptophan (1MT) is available[20]. Recently, a phase I study with 1MT has started for patients with refractory solid tumors (ClinicalTrials.gov identifier: NCT00739609). Following this phase I study, 1MT could be incorporated in phase II studies in AML.
The research we did on the activation of the TRAIL pathway with antibodies directly targeting the pro-apoptotic R2 receptor on AML samples is promising. However, activation of only the extrinsic pathway is presumably not effective enough to kill tumor cells that have already been shaped by the immune system. Many studies have now demonstrated synergistic activity of sTRAIL/Apo2L with conventional chemotherapy[21-24]. These effects are ascribed to the combined activation of the extrinsic and intrinsic apoptotic pathway. Also, synergistic effects of proteasome inhibitors like bortezomib[25], kinase inhibitors[26] with sTRAIL/Apo2L or TRAIL receptor antibodies have been described. Furthermore, the mechanism of the anti-tumor activity of HDAC inhibitors can also be ascribed to upregulation of TRAIL and its agonistic receptors[27]. Fully human antibodies directly targeting R2 have already entered phase I and II studies[25;28] and we are currently exploring the possibility of incorporating an R2 antibody in a phase II trial for AML patients.

In conclusion, this thesis provides insight in strategies that leukemic cells apply to escape from immune surveillance. This knowledge offers many targets for developing immune therapy. In the near future, knowledge about MHC class II

antigen presentation will be incorporated in the research performed to generate vaccines for AML patients. Furthermore, we have planned to further unravel the role of immune surveillance in MDS by closely monitoring immune-modulating new drugs and we currently employ the use of TRAIL targeting human antibodies into the treatment of AML patients.

Reference List

1. van de Loosdrecht AA, van den AW, Houtenbos I, Ossenkoppele GJ, Westers TM. Dendritic cell-based immunotherapy in myeloid leukaemia: translating fundamental mechanisms into clinical applications. Handb.Exp.Pharmacol. 2009;188:319-348.
2. Marijt WA, Heemskerk MH, Kloosterboer FM et al. Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. Proc.Natl.Acad.Sci.U.S.A. 2003;100:2742-2747.
3. Rosenberg SA. Progress in human tumour immunology and immunotherapy. Nature. 2001;411:380-384.
4. Finn OJ. Cancer vaccines: between the idea and the reality. Nat.Rev.Immunol. 2003;3:630-641.
5. Roucard C, Thomas C, Pasquier MA et al. In vivo and in vitro modulation of HLA-DM and HLA-DO is induced by B lymphocyte activation. J.Immunol. 2001;167:6849-6858.
6. Alessandrino EP, la Porta MG, Bacigalupo A et al. WHO classification and WPSS predict post-transplantation outcome in patients with myelodysplastic syndrome: a study from the Gruppo Italiano Trapianto di Midollo Osseo (GITMO). Blood. 2008;112:895-902.
7. Epling-Burnette PK, Bai F, Painter JS et al. Reduced natural killer (NK) function associated with high-risk myelodysplastic syndrome (MDS) and reduced expression of activating NK receptors. Blood. 2007;109:4816-4824.
8. Fauriat C, Just-Landi S, Mallet F et al. Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. Blood. 2007;109:323-330.
9. Chamuleau MED, Feller N, Kelder A, Loosdrecht AA van de, Ossenkoppele GJ. High CLIP (Class II Associated Invariant Chain Peptide) Expression on Minimal Residual Disease Cells Is Associated with High Relapse Rate in AML Patients. [abstract]. Blood (ASH Annual Meeting Abstracts) 108: 2300. 2007;
10. Jadersten M, Malcovati L, Dybedal I et al. Erythropoietin and granulocyte-colony stimulating factor treatment associated with improved survival in myelodysplastic syndrome. J.Clin. Oncol. 2008;26:3607-3613.
11. Chamuleau MED, Zevenbergen A, van Dreunen L ea. Treatment with Epo/G-CSF of patients with low/int-l risk myelodysplastic syndrome diminishes relative CLIP (class II-associated invariant chain peptide) amount on hematopoietic precursor cells. [abstract]. Leukemia Res 2007;31:S61, 2007:
12. Cheuk AT, Chan L, Czepulkowski B et al. Development of a whole cell vaccine for acute myeloid leukaemia. Cancer Immunol.Immunother. 2006;55:68-75.
13. Cheuk AT, Guinn BA. Immunotherapy of acute myeloid leukaemia: development of a whole cell vaccine. Front Biosci. 2008;13:2022-2029.
14. Armstrong TD, Clements VK, Martin BK, Ting JP, Ostrand-Rosenberg S. Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. Proc.Natl.Acad.Sci.U.S.A 1997;94:6886-6891.
15. Armstrong TD, Clements VK, Ostrand-Rosenberg S. MHC class II-transfected tumor cells directly present antigen to tumor-specific CD4+ T lymphocytes. J.Immunol. 1998;160:661-666.
16. Qi L, Rojas JM, Ostrand-Rosenberg S. Tumor cells present MHC class II-restricted nuclear and mitochondrial antigens and are the predominant antigen presenting cells in vivo. J.Immunol. 2000;165:5451-5461.

17. Chamuleau ME, Souwer Y, Van Ham SM et al. Class II-associated invariant chain peptide expression on myeloid leukemic blasts predicts poor clinical outcome. Cancer Res. 2004;64:5546-5550.

18. Morimoto Y, Toyota M, Satoh A et al. Inactivation of class II transactivator by DNA methylation and histone deacetylation associated with absence of HLA-DR induction by interferon-gamma in haematopoietic tumour cells. Br.J.Cancer. 2004;90:844-852.

19. Lyko F, Brown R. DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. J.Natl.Cancer Inst. 2005;97:1498-1506.

20. Hou DY, Muller AJ, Sharma MD et al. Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. Cancer Res. 2007;67:792-801.

21. Mizutani Y, Nakanishi H, Yoshida O et al. Potentiation of the sensitivity of renal cell carcinoma cells to TRAIL-mediated apoptosis by subtoxic concentrations of 5-fluorouracil. Eur.J.Cancer 2002;38:167-176.

22. Ashkenazi A, Herbst RS. To kill a tumor cell: the potential of proapoptotic receptor agonists. J.Clin.Invest. 2008;118:1979-1990.

23. Wu XX, Kakehi Y, Mizutani Y et al. Enhancement of TRAIL/Apo2L-mediated apoptosis by adriamycin through inducing DR4 and DR5 in renal cell carcinoma cells. Int.J.Cancer 2003;104:409-417.

24. Wu XX, Kakehi Y, Mizutani Y et al. Doxorubicin enhances TRAIL-induced apoptosis in prostate cancer. Int.J.Oncol. 2002;20:949-954.

25. Georgakis GV, Li Y, Humphreys R et al. Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. Br.J.Haematol. 2005;130:501-510.

26. Rosato RR, Almenara JA, Coe S, Grant S. The multikinase inhibitor sorafenib potentiates TRAIL lethality in human leukemia cells in association with Mcl-1 and cFLIPL down-regulation. Cancer Res. 2007;67:9490-9500.

27. Nebbioso A, Clarke N, Voltz E et al. Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. Nat.Med. 2005;11:77-84.

28. Humphreys RC, Halpern W. Trail receptors: targets for cancer therapy. Adv.Exp.Med.Biol. 2008;615:127-158.