1. GENERAL HYPOTHESIS AND IMPLICATIONS

In patients with AML, leukemic cells constantly encounter T cells in the peripheral blood and lymphoid tissues. Therefore, in the present thesis, we approached the pathogenesis of AML as an immunological problem, where leukemic cells contain specific capabilities to abrogate leukemia-specific T cell function, leading to excessive outgrowth during disease onset and MRD followed by overt relapse. We propose that such capabilities of leukemic cells can cause excessive outgrowth via indirect and direct effects on T cell activation. As an indirect effect, the increasing numbers of leukemic cells during the acute phase of the disease may result in ineffective T cell priming by creating an immunosuppressive environment via tumor immune editing, which leads to inactivation of professional APCs, or generating competition for these APCs to interact with and activate T cells in the lymph nodes. Alternatively, we have previously found indications that leukemic cells make use of escape mechanisms that directly interfere with priming and/or recognition of T cells (Figure 1), either by upregulating IDO expression to cause T cell suppression, or overexpressing TRAIL-R3 as decoy receptor to prevent T cell-mediated apoptosis.

2. SUMMARY OF RESULTS

Dendritic cells as APCs in AML (part II)

Because of an apparently inadequate immune response generated against leukemic cells in vivo, vaccination with LAA-containing donor APCs is an attractive method to boost leukemia-specific T cell immunity in AML patients. We explored whether DCs could be prepared ex vivo and their potential to become efficient LAA-presenting APCs for use as potential cellular vaccines. In Chapter 2, an overview of current literature underlines that culturing DCs from monocytes and loading these so-called moDCs with exogenous LAAs holds great promise as immunotherapeutic strategy. In contrast to leukemia-derived DCs, moDCs can be produced on a large scale and may be broadly applicable to patients if activation and specificity against leukemic cells can be introduced. As a limited number of patients have been tested for this protocol, additional investigation is necessary to establish which type of LAA loading strategy in combination with or without adjuvants is the most effective. Chapter 3 describes one of those studies, in which moDCs were cultured and functionally compared after loading with leukemic cell lysates or apoptotic leukemic cells of AML patients and subsequent stimulation by a maturation-inducing cytokine cocktail (consisting of IL-1β, IL-6, TNF-α and PGE₂) and the clinically applicable TLR7/8 ligand R848. We show that heat shock-induced apoptotic leukemic cells are more efficiently taken up than leukemic cell lysates and that this could be enhanced in the presence of R848. Furthermore, the addition of R848 improved the quality of antigen presentation by LAA-loaded DCs, as determined by reduced CLIP presentation, and their ability to migrate and stimulate T cell proliferation. Unfortunately, the sequential use of R848 and the maturation cocktail reduced both the migratory and T cell stimulatory capacity and suppressed IL-12p70 production of loaded DCs, indicating that other TLR ligands need to be investigated in an attempt to maintain effective maturation of moDCs after loading with apoptotic leukemic cells.
Leukemic cells as APCs in AML (part III)

Using myeloid leukemic cell lines and patient material, we examined leukemic cells for their role as APCs and focused on CLIP expression as possible tumor escape mechanism in AML by directly interfering with T cell priming and recognition. In chapter 4, the analysis of CLIP on leukemic cells in an expanded cohort of newly diagnosed AML patients confirmed that high expression indeed is significantly correlated to a shortened OS and DFS. Moreover, by down-modulation of CLIP with li siRNA, we provide evidence that the clinical impact of CLIP can be explained by its effect on T cell activation. Allogeneic donor CD4+ T cells revealed high proliferation rates during MLRs with CLIP down-modulated cells from Kasumi-1 and THP-1 myeloid leukemic cell lines, as compared to CLIP- wild-type cells from the same cell lines. By using freshly obtained AML samples in chapter 4 and 5, we further disclose that absence of CLIP on primary leukemic cells stimulates autologous CD4+ T cells in favour of a leukemia-specific immune response. Besides the viability and activation (CD25+/HLA-DR+) of CD4+ T cells, also their skewing towards Th1 (IFN-γ, effector memory (CD27/CD45RA) as well as antigen-specific (TCR-Vβ restricted) phenotypes was enhanced in CLIP co-cultures, in contrast to CLIP- co-cultures of the same AML patients. Additionally, outgrowing CD4+ T cells from CLIP co-cultures showed reactivity to autologous CLIP leukemic cells, but not to autologous non-malignant monocytes, which strongly suggests a negative role for CLIP on primary leukemic cells in LAA-specific CD4+ T cell induction. We also found implications for a similar involvement of CLIP on residual leukemic cells from treated AML patients, as shown in chapter 6. In patients who encountered a relapse, CLIP was significantly more prevalent on LAP-positive blasts as compared to non-relapsing patients. Interestingly, in those patients who were considered as MRD-negative, high CLIP expression was predictive for a shortened RFS, which indicates that CLIP could be useful as a marker for MRD evaluation and might be involved in the immune escape of leukemic cells during AML recurrence. The finding in chapter 5 that ‘CLIP-primed’ CD4+ T cells could react to CLIP- leukemic cells, but not to non-malignant monocytes, suggested that CLIP expression correlated with reduced expression of antigens that cause leukemia-specific T cell activation. According to the classical antigen presentation route in APCs, this is caused by decreased loading of antigens onto HLA class II in the MIICs, a process regulated by the function of DM and DO. We also found evidence for this in leukemic cells of AML patients, as high CLIP expression was associated with low DM/DO expression ratios. Another possible explanation is that in CLIP- leukemic cells, antigen loading is already determined in the ER, where association of li with newly synthesized HLA class II molecules prevents endogenous (potentially leukemia-associated) antigens. Indications for this notion are shown in chapter 7, in which two key participants of the endogenous antigen loading pathway appear to be involved in HLA class II antigen presentation on CLIP KG-1 and ME-1 leukemic cell lines. li silencing did not affect HLA-DR transport to the plasma membrane, whereas suppression of the proteasome and TAP resulted in lowered expression of HLA-DR. This was not observed for CLIP+ Kasumi-1 cells, which might implicate a possible other relationship between CLIP expression and HLA class II-mediated endogenous antigen presentation than the classical exchange of CLIP for antigens in the MIICs. Strikingly, as demonstrated in chapter 8, we noticed abundant CLIP expression on leukemic cells from patients with APL, a subtype of HLA-DR-negative AML genetically characterized by t(15;17). Among standard used markers for APL classification, CLIP was the most significant marker that distinguished APL from other HLA-DR+ non-APL AML patients. A correct classification of APL was achieved for all AML cases.
according to CLIP analysis using flow cytometry alone. To our knowledge, CLIP is revealed here as the first positive marker for APL. In addition to its use in flow cytometric identification of HLA-DR AML, we explored the underlying mechanism for this type of CLIP expression on leukemic cells. In chapter 9, we show that CLIP can alternatively be involved in the HLA class I antigen presentation pathway of leukemic cells. Western blot analyses of total lysates and immunoprecipitates revealed the direct association of Ii proteins with HLA class I molecules in myeloid leukemic cell lines and AML patients. Furthermore, we found several Ii peptides in HLA class I-specific eluates of B-LCLs, of which two were located within the CLIP region. In peptide binding assays, several CLIP peptides further showed strong binding of CLIP to several HLA class I alleles containing completely distinct antigen-binding grooves, including HLA-A2, -B7, -A3 and -B40. This demonstrates that CLIP is able to promiscuously bind to HLA class I, similar to HLA class II, and thus might interfere with HLA class I antigen presentation on leukemic cells as well.

3. DISCUSSION AND FUTURE PERSPECTIVES

The results from this thesis provide evidence that aberrant LAa processing and presentation can be involved in the immune escape of leukemic cells in AML. The application of moDCs loaded with exogenous LAAs as cellular vaccines (discussed in chapter 2 and 3) might have several advantages related to other currently tested immunotherapeutic strategies. It is a tool that could not only be generally useful for AML patients due to the high DC production rate in culture and efficient presentation of a broad range of LAAs, but may also contribute to the evasion of the inhibitory effects exerted by leukemic cells on LAa-specific T cell activation (Figure 5C and D). In addition, we show that CLIP expression on leukemic cells is associated with poor prognosis (chapter 4 and 6) and directly reduces T cell function (chapter 4 and 5) in AML, suggesting that either an impaired presentation of endogenous LAAs or increased CLIP expression on leukemic cells is involved in AML immunopathogenesis by interfering with leukemia-specific T cell immunity. The mode of regulation and action of CLIP expression and its potential as a target for future immunotherapeutic applications in AML will be further discussed below.

New insights into mechanisms that underlie CLIP expression

In CLIP leukemic cells, we revealed that HLA class II antigen presentation can be dependent on processing of endogenous antigens by the proteasome and TAP (chapter 7), indicating an alternative antigen presentation pathway in leukemic cells involving interaction of HLA class II molecules with endogenous antigens. Because siRNA-induced Ii silencing did not influence HLA-DR presentation by CLIP leukemic cells, it is most probable that in this pathway, newly synthesized HLA class II molecules are loaded with endogenous antigens instead of Ii in the ER and are directly transported from the Golgi apparatus to the plasma membrane (Figure 4B and ref. 5;6). Still, it is possible that HLA-DR complexes enter the endosomal/lysosomal pathway via internalization from the plasma membrane and are recycled after reloading with endogenous antigens in the MIICs.7;8 We further showed that CLIP presentation can occur independently of HLA class II processing (chapter 8) and Ii binds to HLA class I molecules (chapter 9) in leukemic cells. This implicates that a second alternative antigen presentation pathway can be active in leukemic cells as well, which might prevent endogenous antigen presentation by HLA class I
molecules (Figure 4A). Most indications for this pathway were observed in TAP-deficient cells (T2, THP-1, Kasumi-1), indicating that a lack of endogenous peptides translocated into the ER is a requisite. It is therefore likely that binding of II instead of endogenous antigens to newly formed HLA class I molecules in the ER is the underlying process. The next question to address is whether complexes of HLA class I and II are directly transported to the plasma membrane or, similar to classical HLA class II processing, first directed to the MIICs for further processing of CLIP and loading with endogenous antigens.

The potential involvement of CLIP in HLA class I antigen presentation by leukemic cells has extended our ideas about its immunoregulatory function in AML. First of all, it might give an additional explanation for the strong clinical impact of CLIP in patients (chapter 4), as its presentation on leukemic cells by HLA class I molecules could suppress leukemia-specific CD8+ CTL-mediated elimination. To test the functional effect of CLIP in this context, CTL co-cultures with autologous CLIP+ and CLIP- primary leukemic cells have to be performed in a similar way as in chapter 5. Secondly, the ratio of CLIP to DR expression as an indicator for HLA class II antigen presentation by leukemic cells could be biased by its role in HLA class I processing. The function of the classical mediators of endogenous antigen processing now have to be taken into account for HLA class II antigen presentation as well. According to our cell line studies, there also seemed to be an association of intracellular TAP, II and DM with surface CLIP expression by
leukemic cells. CLIP leukemic cells showed increased levels of TAP and DM and reduced levels of Ii, as compared to CLIP+ leukemic cells. This implies crosstalk of HLA class I and II antigen presentation pathways and a general mechanism for impaired LAA presentation in leukemic cells. Therefore, presence of gene abnormalities in the master regulator of HLA class II antigen presentation, CIITA, in leukemic cells may be the major cause for such aberrancy, which should be investigated in AML patients.

The potential of CLIP expression as immunotherapeutic target in AML

Our observations on alternate HLA class I and II antigen presentation by leukemic cells indicate that CLIP expression could be a primary reason for the lack of anti-leukemic T cell immunity in patients with AML, both before and after DC-based tumor immunotherapy. In a proposed AML-T cell model, CLIP leukemic cells should be well-recognized and eradicated by presenting endogenous LAAs to LAA-specific CD4+ T cells and/or CTLs (Figure 5A). This initiates a potential feed-forward loop wherein exogenous LAAs are taken up by professional APCs, such as DCs, and presented for priming of LAA-specific T cells. Thus, in CLIP AML patients, only LAA-specific T cell priming may be suboptimal, which makes vaccination with LAA-loaded DCs a good treatment option. In CLIP AML patients however, CLIP expression on leukemic cells indicate deficient HLA class I- and/or HLA class II-mediated presentation of endogenous LAAs. This can put the feed-forward loop on hold, either directly by preventing their recognition and eradication by T cells, or indirectly by creating an immunosuppressive environment that causes functional inactivation of DCs or T cells, resulting in impairment of T cell priming (Figure 5B). A third possibility is that LAA-specific T cell priming solely depends on LAA presentation by leukemic cells and is directly affected by CLIP expression, but this seems less likely. Administration of LAA-loaded DC vaccines to these patients may establish LAA presentation to stimulate T cell priming, however it does not deal with the disturbing role of CLIP in endogenous LAA presentation by leukemic cells, which then still leads to impaired T cell recognition (Figure 5C) and their uncontrolled outgrowth. The presence of CLIP could even have an additional, direct inhibitory effect on T cells. As described for maturing DCs,9 increased levels of CLIP antagonized the priming of autologous Th1 cells, indicating its role as self peptide in immune tolerance. If CLIP has the same impact on leukemic cells, a self-tolerant, but also leukemia-protective T cell environment might be generated in CLIP+ AML patients. In accordance with our implications in chapter 5, it is possible that it acts together with CD86 to prime Th2 cells or Tregs and promote hyperleukocytosis.10 In either way, we postulate that CLIP expression on leukemic cells could be one of the causes for the poor responses to DC-based immunotherapy in AML until now.

CLIP down-modulation as immunotherapeutic strategy in AML

To circumvent this type of immune escape by leukemic cells, additional immunotherapeutic strategies need to be included in current protocols that down-modulate CLIP in vivo (Figure 5D). The design of such strategies may have great promise for future active immunotherapy by simultaneously preventing the possible negative influence of CLIP on HLA class I and II antigen presentation. CLIP leukemic cells then become more ‘visible’ to LAA-specific CD4+ T cells and CTLs, and those that display costimulatory molecules (e.g., CD86) are additionally triggered to prime instead of inhibit T cells (Figure 5C and D). Furthermore, as a result, LAA epitope
Figure 5. Concept for CLIP expression as tumor immune escape mechanism during immune surveillance and active immunotherapy in AML. Situations before and after immunotherapy in AML patients are proposed: (A) Tumor immunity in untreated CLIP- AML; LAA-specific T cell priming and recognition are optimal due to enhanced endogenous LAA presentation by leukemic cells. (B) Tumor immune escape in untreated CLIP+ AML; leukemia-specific T cell priming as well as recognition are hampered because of an indirect and direct role of CLIP in LAA presentation by myeloid DCs and leukemic cells, respectively. (C) Tumor immune escape in treated CLIP+ AML; although priming of leukemia-specific T cells is resolved by DC vaccination, leukemic cells still escape their recognition by expressing CLIP. (D) Tumor immunity in treated CLIP+ AML; by using DC vaccination and in vivo immunomodulatory drugs, both T cell priming and recognition might be targeted, which induces a potent immune response against leukemic cells.
spreading might occur, which leads to the introduction of new epitopes that induce a broad leukemia-specific T cell response.

One possible strategy to realize CLIP down-modulation is to potentiate the processing machinery of endogenous antigens, such as the proteasome and TAP. As shown in chapter 3, offering exogenous LAAs from apoptotic material to immature moDCs already resulted in decreased CLIP expression, indicating that presence of LAAs alone is sufficient for efficient presentation by these well-equipped APCs. A second candidate strategy to decrease CLIP expression is to boost loading machinery for endogenous antigens in the mIICs. In line with the decreased DM/DO expression ratio found in CLIP+ leukemic cells (chapter 4 and ref. 4) and potential processing of endogenous antigens via autophagy in the mIICs (Figure 5), the function of DM and/or DO is an attractive target to stimulate this type of antigen processing. Suppression of protein kinase C (PKC) was reported to affect DO expression in activated B cells,11 potentially leading to a decline in CLIP, which points to the use of PKC inhibitors in CLIP+ AML. As mentioned earlier, in leukemic cells having limited equipment to process and present LAAs via the classical antigen presentation route (e.g., low II and DM expression), intact endogenous antigen machinery (e.g., TAP) seemed to be inversely correlated to CLIP expression (chapter 7 and 9). This may imply that when endogenous LAAs processing is deficient, II instead of TAP-associated peptides can interact with either newly synthesized HLA class I or HLA class II molecules in the ER. For this type of antigen processing, we speculate that the ratio between TAP and II expression levels could be the deciding factor for antigenic peptide loading to occur in the ER or MIICs, respectively. Administration of clinically applicable histone deacetylase inhibitors (HDACi) might be a powerful tool in this case, as it can enhance expression of TAP subunits in tumor cells.12,13 Indeed, we very recently found a strong down-modulation of CLIP on leukemic cells from AML patients after treatment with the HDACi suberoylanilide hydroxamic acid (SAHA; unpublished data).

CLIP down-modulation versus DC vaccination in AML immunotherapy
The road to eventually accept CLIP down-modulation as an immunotherapeutic modality in AML, however, will not be without obstacles. CLIP is also expressed by normal cells, such as DCs, B cells and thymic epithelial cells,9,14,15 which may raise the chance of autoimmunity. Nevertheless, some HDACi are able to suppress autoimmune manifestations,16 while others induce immune gene expression in tumor cells,17 and are already tested in clinical trials for solid and hematological cancers, including AML.18 Thus, the influence of autoimmunity by targeting CLIP will also be dependent on the selectivity of these and other modulators and needs further investigation. Another problem that has to be overcome is the heterogeneity of the disease. From an immunological point of view, this leads to a wide variety of aberrant immunological processes between patients, probably as the result of tumor immune escape mechanisms affecting the immunogenicity of DCs and leukemic cells in different ways.

As a first step to overcome these difficulties, answers to certain questions could assist in predicting if DC vaccination and/or immunomodulation, including CLIP down-modulation, will be effective as immunotherapeutic strategy for patients during MRD. Based on our general model for impaired LAAs presentation in untreated AML (Figure S5A and B), these questions regard the ability of both host DCs and leukemic cells to activate LAAspecific T cells (Figure 6). In AML patients with well-functioning DCs, vaccination with LAAsloaded DCs may not be needed for LAAspecific
Figure 6. Main research questions to be resolved to better understand the role of antigen presentation in AML and to select specific strategies for optimal active immunotherapy in patients.

T cell priming, as the negative effect by circulating leukemic cells is probably overruled. Still, an optimal ‘recognition profile’ for leukemic cells is needed for their specific elimination by CTLs. This means that HLA class II expression and costimulation on leukemic cells may be neglected, but for those expressing CLIP on HLA class I, endogenous LAA processing and presentation has to be upregulated in vivo. If the function of host DCs is not optimal, priming of leukemia-specific Th1 cells and CTLs has to be established by LAA-loaded DC vaccines, presenting exogenous LAAs, and preferably also by leukemic cells with a ‘priming profile’, presenting costimulatory molecules, HLA class II as well as endogenous LAAs. Such a profile has the potential benefit that T cells are primed to respond against the same LAAs as expressed on leukemic cells. To establish this, not only CLIP has to be down-modulated, but also other factors involved in the immunogenicity of leukemic cells must be optimal. Therefore, by further elucidation of the lack of anti-leukemic T cell immunity in AML, well-thought choices for application of DC vaccination and/or immunomodulation of leukemic cells can be made in the future, which should increase the chance of inducing a potent anti-leukemic T cell response in vivo.

Other immunomodulatory strategies to enhance antigen presentation in AML

In addition to these general choices, progress has to be made in the design of strategies that can optimize leukemia-specific immunogenicity in AML patients. Since we showed a negative role for CLIP expression on leukemic cells regarding this issue, it may be considered to put more effort into research that aims to specifically enhance LAA presentation in vivo. Likely, due to the heterogeneity of the disease, this has to be addressed at several levels of antigen presentation, including expression of HLA and costimulatory molecules, LAA processing and LAA presentation (Figure 7).

Concerning DC vaccines, we showed that moDC loading with apoptotic leukemic material is a promising strategy, since their maturation resulted in increased HLA class II expression,
costimulation and antigen presentation (chapter 3). To further increase LAA-specific loading, TLR ligands other than R848 have to be investigated for their effect on maturation of loaded moDCs. For leukemic cells, not only CLIP can be overexpressed, but also the expression of HLA class II and costimulatory molecules and the function of endogenous antigen processing machinery can be impaired. For example, leukemic cells that do not express HLA class II or costimulatory molecules can be differentiated ex vivo into leukemic DC-like cells, serving as whole cell vaccines. Further treatment options for such type of vaccines may be lentiviral introduction of CIITA, B7 or 4-1BB genes, serum starvation or treatment with li siRNA (Figure 7). Alternative and maybe more feasible strategies to modulate LAA processing and presentation in vivo are the addition of IFN-γ and the use of HDACi and PKC inhibitors, as discussed earlier. Since some HDACi inhibitors have been reported to negatively affect DC

Figure 7. Potential approach using immunomodulatory strategies to acquire potent LAA-presenting APCs in AML. Knowledge about the immunogenicity of DCs and leukemic cells could improve active immunotherapy by specifically choosing optimal immunomodulatory strategies for each patient. Strategies are suggested that may be of value for increasing HLA class II expression and costimulation (1), LAA processing (2) or LAA presentation (3) on moDC and whole-cell vaccines ex vivo as well as leukemic cells in vivo. Of note, leukemic cells constitutively display HLA class I. APC, antigen-presenting cell; CC, cytokine cocktail; CIITA, class II transactivator; CLIP, class II-associated invariant chain peptide; HDAC, histone deacetylase; li, invariant chain; LAA, leukemia-associated antigen; TLR-L, Toll-like receptor ligand; PKC, protein kinase C.
immunogenicity as well, caution has to be exercised when using such immunomodulatory drugs as immunotherapeutic treatment. Eventually, based on experimental results, effective strategies have to be selected to develop a feasible and easy-to-use treatment protocol to enhance LAA presentation in AML patients.

4. OVERALL MESSAGE

Further analysis of aberrant LAA presentation at the time of diagnosis and upon relapse of the disease could give new insights in AML progression and the potential failure of current active immunotherapy. One of the mechanisms that should be further unravelled is the role of CLIP and Ii in the HLA class I antigen presentation pathway. Abundance of HLA class I on leukemic cells might be an underestimated occurrence, since LAA presentation is probably disturbed by abnormal interaction of Ii and CLIP with HLA class I molecules, leading to CTL escape. This, together with its adverse role in the context of CD4+ T cells, strengthens CLIP as target for immunomodulatory strategies in AML. The final goal towards the development and use of such immunotherapeutic tools should include the design of an efficient monitoring system by which impaired LAA presentation can be characterized during both diagnosis and follow-up to choose the best-suited tailor-made strategy for improving tumor immunogenicity in each individual AML patient.

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