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Targeting the Minimal Residual Disease in Acute Myeloid Leukemia: The Role of Adoptive Immunotherapy with Natural Killer Cells and Antigen-Specific Vaccination

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1. Introduction

Acute myeloid leukemia (AML) is a neoplastic disorder characterized by the clonal expansion of non-lymphoid hematopoietic progenitor cells with failure of normal hematopoiesis. Several biological and clinical parameters have been identified at diagnosis to classify different AML subtypes with different prognosis. In this view, genetic abnormalities confer the most important prognostic information. Therapeutic interventions based on conventional or high-dose chemotherapy have significantly improved the complete remission (CR) rates of acute leukemia. However, a significant portion of responding patients still harbors a minimal residual disease (MRD), which is often resistant to further pharmacological treatments and ultimately leads to disease relapse and progression. Although allogeneic stem cell transplantation may significantly improve the clinical results of AML patients who achieved complete remission, such approach has several and important limitations and is not applicable to all the patients. For these reasons, novel therapeutic approaches to improve the clinical outcome of AML patients are under investigation, and treatments with high compliance such as adoptive and active immunotherapy are desirable. Aim of the present work will be to focus the most relevant insights in the field. In particular, we will report about the use of natural killer (NK) cells as a means of adoptive immunotherapy against neoplastic cells, including AML. Moreover, we will discuss the role of vaccines against leukemia with particular emphasis on the immunogenicity of novel and promising leukemia-associated antigens.

2. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a hematopoietic malignant disease rising from neoplastic transformation of myeloid stem cells. This causes the alteration of the normal cell differentiation and proliferation systems, resulting in the accumulation in bone marrow and peripheral blood of non-functional myeloid cells termed myeloblasts. AML may arise *de novo* or secondary to pre-existing myelodysplasia or previous chemotherapies.

Myeloblasts lack the normal proliferation systems and their over-proliferation and accumulation in bone marrow and peripheral blood cause lack of production of hematopoietic normal cells, this resulting in peripheral deficiency of platelets, neutrophils and hemoglobin.

Prognosis of AML depends on multiple factors: age at diagnosis (age>60 years is a poor prognostic factor), hyperleukocytosis, cytogenetic status and molecular specific characteristics are the most important ones.

AML can occur at every age, but its incidence increases with age (median age at presentation: 65 years). It has an annual incidence of 3.6 per 100,000. This incidence increases with age, rising to 16.3 per 100,000 per year in the over 65 age group. Older adults typically have a highly inferior prognosis and an increased risk of therapy-related toxicity and mortality.

Conventional treatment of AML is based on chemotherapy regimens and consists of several well-defined phases: the first one is the CR-induction cycle, based on the administration of 3 days-anthacycline associated with 7 days-cytarabine. Its aim is to ‘empty’ bone marrow and allow the normal hematopoietic cells repopulation.

Response rates with conventional chemotherapy range from 60% to 85% in young adults (age < 60 years), but more than 50% of these patients are going to relapse, with a five-year overall survival of 40%. Older patients with a diagnosis of AML have a poorer prognosis, with less than 10% of long survivers; this is due to biological unfavorable risk factors, such as unfavorable cytogenetics, which are more frequent in the elderly (Leith et al. 1997).

One of the main cause of relapse in patients who achieved complete remission after chemotherapy is the persistance of a small amount of leukemic cells termed MRD. Minimal residual disease detection became one of the main tasks for hematologists; immunophenotypical and molecular markers able to discriminate normal cells from blastic cells allow the detection of residual leukemic cells not detected by morphologic examinations.

After the induction therapy two or more consolidation cycles are needed in order to eradicate leukemic cells completely. Allogeneic stem cell transplantation is one of the most effective consolidation therapy, even if it is feasible only for fit patients and only if a suitable HLA-matched donor is available.

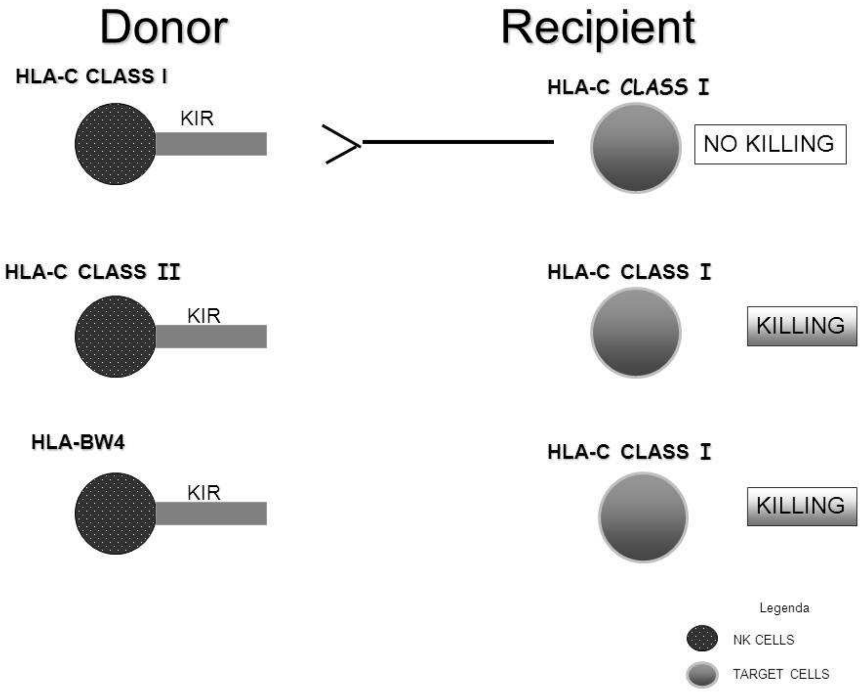
Moreover, allogeneic stem cell transplantation is highly effective if performed after obtaining first CR, while its efficacy is poor in case of relapsed/refractory patients.

Only young and fit patients can undergo stem cell transplantation because of its significantly high toxicity, mortality and morbidity rates.

Attempts to effectively prime and sustain anti-tumor immunity against leukemic cells have recently provided promising preclinical and clinical results. Results from allogeneic stem cell transplantation (SCT) represent the main evidence that leukemic cells are targets of the immune system. In fact, since the first clinical observation that allogeneic SCT offered a clinical advantage over autologous transplantation due to a graft-versus-leukemia (GVL) effect, much more attention has been given to the role of adoptive immunotherapy over conditioning regimen as a means to eradicate tumor cells. In particular, donor lymphocyte infusions (DLIs) are capable to restore a durable complete remission. Such results are the proof of principle of the crucial activity of anti-tumor immunity in controlling the growth of leukemic cells.

3. Adoptive immunotherapy with natural killer cells

Human NK cells are a subset of PB lymphocytes defined by the expression of CD56 or CD16 and the absence of the T-cell receptor (CD3) (Robertson et al, 1990). They recognize and kill transformed cell lines in an MHC-unrestricted fashion and play a critical role in the innate immune response. Several studies demonstrated that NK function, which is distinct from the MHC-restricted cytolytic activity of T cells, may be relevant for the immune control of tumor development and growth. Although NK cell killing is MHC-unrestricted, NK cells display a number of activating and inhibitory receptors that ligate MHC molecules to modulate the immune response (Lanier et al, 1998). NK cell receptors that recognize antigens at the HLA-A, -B, or -C loci are members of the immunoglobulin super family and are termed killer immunoglobulin receptors or KIRs (Farad et al, 2002). Engagement of these NK cell receptors results in stimulation or inhibition of NK cell effector function, which ultimately depends on the net effect of activating and inhibitory receptors (Figure 1).



Clinical trials attempting to utilize the anti-tumor effect of NK cells have met only modest success due to the lack of understanding of receptors and ligands which determine whether NK cells will be activated or suppressed. On the contrary, data from haploidentical T-cell depleted transplantation suggest that KIR mismatch with tumor MHC may significantly impact on tumor cell killing, particularly in AML (Ruggeri et al, 2002). In fact, these studies show that AML patients with KIR ligand mismatch are significantly protected against leukemia relapse. In addition, preclinical and clinical investigations demonstrated that haploidentical KIR-mismatched NK cells play the main role as anti-leukemia effector cells and they exert their cytotoxic activity within 4-5 days (Ruggeri et al 2002, Ruggeri et al, 1999). In particular, high risk AML patients with a KIR-ligand mismatch in the graft-versus-host (GVDH) direction had a relapse rate of 0% compared to KIR-ligand matched patients who had a relapse rate of 75%. Given these results, haploidentical KIR-mismatch NK cells administered to AML patients as cell-based immunotherapy may induce NK cell-mediated killing of leukemia cells resulting in the elimination of residual disease in high risk AML patients. Furthermore, alloreactive mismatched NK cells facilitate hematopoietic engraftment after infusion of haploidentical stem cells, and inhibit the onset of GVHD by targeting host antigen-presenting cells (Ruggeri et al, 2002). Of note, the differential expression of activating ligands on hematopoietic and not hematopoietic tissues may provide an additional explanation for the observed GVL effect in the absence of GVHD.

Partially purified haploidentical NK cells have been already used clinically and labeled with ^{111}In to track, in vivo, their kinetics and organ distribution in patients with renal cancer (Brand et al, 2004). A seminal study demonstrated that up to 1.5×10^7/haploidentical NK cells/Kg can be safely infused in AML and cancer patients following Fludarabine/ Cyclophosphamide (Flu/Cy) immunosuppressive chemotherapy and, in some cases, clinical responses without GVHD had been observed (Miller et al, 2005). Interestingly, circulating haploidentical NK cells were found, in selected patients, up to 28 days after infusion especially when exogenous IL-2 was given for 9 doses. In vivo expansion of NK cells was correlated with a high IL-15 serum concentration. In particular, in this study, 19 poor risk AML patients were reported who had received a cell population containing a median of $8.5 \pm 0.5 \times 10^6$ and $1.75 \pm 0.3 \times 10^5$ NK and T cells, respectively. Five out of 19 patients achieved CR. NK cells adoptive immunotherapy was well tolerated and hematological and non hematological toxicity were mainly related to the immunosuppressive regimen and IL-2 administration. The maximum tolerated dose of NK cells was not achieved and GVHD was not observed despite the relatively high number of haploidentical T cells infused. However, it should be noted that NK cells were only partially purified after a single round of depletion of $CD3^+$ cells which resulted in less than 2 logs reduction of T cells.

More recently, a study of haploidentical KIR-HLA mismatched NK cell transplantation in childhood AML reported that NK cell therapy prolonged disease-free and overall survival (Rubnitz et al, 2010). In this pediatric cohort of AML patients, who underwent NK therapy after an immunosuppressive regimen, the 2-year event-free survival was 100%. Notably, all the children were considered at low-risk of relapse, with a significant fraction harboring good-prognosis cytogenetics. Furthermore, as children weigh less than adults, the median number of infused NK cells was significantly higher than in adult trial and the separation procedure consisted in highly purified NK cells. These differences may partially explain the discrepancy in clinical results and suggest that in adult patients the clinical effect of NK therapy may be implemented by increasing the number of infused NK cells.

We recently published the results of a clinical trial of adoptive immunotherapy with haploidentical KIR-mismatched NK cells in elderly patients with AML (Curti et al, 2011).

Thirteen AML patients, 5 with active disease, 2 in molecular relapse and 6 in morphological complete remission (CR);(median age 62 years, range 53-73) received highly purified $CD56^+CD3^-$ NK cells from haploidentical KIR-ligand mismatched donors after fludarabine/ cyclophosphamide immunosuppressive chemotherapy, followed by IL-2. The median number of infused NK cells was 2.74 x 10^6/Kg. T cells were under 10^5/Kg. No NK cell-related toxicity, including GVHD, was observed. One of the 5 patients with active disease achieved transient CR, whereas 4/5 patients had no clinical benefit. Both patients in molecular relapse achieved CR which lasted for 9 and 4 months, respectively. Three/6 patients in CR are disease-free after 34, 32 and 18 months. After infusion, donor NK cells were found in the peripheral blood of all evaluable patients (peak value on day 10). They were also detected in bone marrow in some cases. Donor-versus-recipient alloreactive NK cells were demonstrated *in vivo* by the detection of donor-derived NK clones that killed recipient's targets. Adoptively transferred NK cells were alloreactive against recipient's cells, including leukemia. Taken together, these data demonstrate that infusion of purified NK cells is feasible in elderly patients with high risk AML.

4. Vaccination against acute myeloid leukemia: WT1 as a novel promising antigen

During the last years a number of studies have demonstrated that tumor-associated antigens (TAA) may be recognized by the immune system leading to the activation of tumor-specific cytotoxic T lymphocytes (CTLs) with the potential to eradicate tumor cells. Moreover, during the last decade the role of dendritic cells (DCs) as natural adjuvants of immune response has been deeply elucidated. The identification of a wide number of TAA, together with new insights into the mechanisms underlying the activation of anti-tumor immune response, has led to the development of novel anti-tumor vaccination strategies which are currently under investigation in the clinical setting. In AML, some TAA, such as PRAME, Wilms' tumor gene (WT1), proteinase 3 have been recently identified. In particular, WT1, which is a zinc-finger transcription factor expressed during normal ontogenesis, is significantly over-expressed in acute and chronic myeloid leukemia and myelodysplastic syndromes and it appears as an attractive target for immunotherapy.

WT1-specific antibodies against the N-terminus portion of the WT1 protein have been found in the sera of AML patients, but not in healthy donors, suggesting that anti-WT1-specific immune response is present in these patients. Preclinical studies have clearly demonstrated that peptides from WT1 may be used to generate in vitro a WT1-specific cytotoxic response (Li et al, 2005; Pinilla-Ibarz et al, 2006; Oka et al, 2000). While a number of WT1-derived CD8 T-cell epitopes have been reported, two peptides, namely HLA-A0201-restricted peptide 126–134 and HLA-A24-restricted peptide 235–243, have been studied extensively. Since murine and human WT1 are similar, WT1 126 and WT1 235 have also been tested in animal models. B6 mice were injected with WT1 peptides and analyzed for the induction of a T-cell and B-cell mediated immune responses against WT1. This analysis revealed that WT1 vaccination induced WT1-specific immunity, which was also capable to delay in vivo the growth of tumor cell lines, naturally overexpressing WT1. Recently, a National Cancer Institute Pilot Project assigned to WT1 the position of best and most suitable target antigen for cancer immunotherapy, due to a number of characteristics, such as its therapeutic function, immunogenicity and expression level (Cheever et al, 2009)

These data have prompted several groups to investigate the role of WT1 as a tumor-antigen in the clinical setting of cancer immunotherapy. Particularly, Oka et al conducted phase I clinical trials using peptide WT1-235 (CMTWNQMNL) and its analogue (CYTWNQMNL) for patients with overt leukemia from MDS, MDS with myelofibrosis and AML (Oka et al, 2004). Vaccination was performed by injecting 0.3–3 mg of native or analogue peptide 235 emulsified with the adjuvant Montanide ISA51. The vaccination resulted in an increase in WT1-specific CTLs followed by a rapid reduction in leukemic blast cells. No serious toxicity was observed, but leukemic blasts relapsed after the vaccination was stopped. Recently, these investigators reported that biweekly injection of AML patients with either native or analogue peptide 235 along with Montanide and GM-CSF resulted in three patients remaining in CR for 4 years (Tsuboi et al, 2007). The WT1-235 peptide has also been shown to induce HLA-A0201-restricted CTL (Pinilla-Ibarz, 2006). Keilholz et al first reported that vaccination of a patient with recurrent AML, using HLA-A0201-restricted WT1 peptide 126 along with KLH as an adjuvant, induced CR (Mailander et al, 2004). No haematological or renal toxicities were observed. Rezvani et al reported a phase I clinical trial in patients with AML, CML and MDS, using combined HLA-A0201-binding peptide vaccines from PR1 169–177 and WT1 126–134. Their results show that the emergence of PR1$^+$ or WT1$^+$CD8$^+$ T cells in patients who received WT1 vaccine was associated with a decrease in WT1 mRNA expression, suggesting a vaccine-driven anti-leukemia effect (Rezvani et al, 2008). An analogue to WT1 peptide 126–134 was generated by substituting R for Y at the position 2 anchor motif (named WT1-A1) (Pinilla-Ibarz et al, 2006). This analogue peptide generated a more potent CD8 T-cell response which recognized and lysed WT1$^+$ leukemia cells in vitro. In addition to the HLA-A0201 and -A24 vaccines derived from WT1 protein, Asemissen et al identified a highly immunogenic HLA-A1-binding WT1 peptide (317–327) that is processed and able to induce a CD8 T-cell response in healthy donors and patients with haematological malignancies (Asemissen et al, 2006).

The German group reported about their phase 2 trial of WT1 peptide vaccination in patients with AML and MDS (Keilholz et al, 2009). Vaccination consisted of GM-CSF subcutaneously days 1 to 4, and WT1126-134 peptide and 1 mg keyhole limpet hemocyanin on day 3. Seventeen AML patients and 2 refractory anemia with excess blasts patients received a median of 11 vaccinations. Treatment was well tolerated. Objective responses in AML patients were 10 stable diseases (SDs) including 4 SDs with more than 50% blast reduction and 2 with hematologic improvement. An additional 4 patients had clinical benefit after initial progression, including 1 CR and 3 SDs. WT1 mRNA levels decreased at least 3-fold from baseline in 35% of patients. In 8 of 18 patients, WT1-tetramer$^+$ T cells increased in blood and in 8 over 17 patients in bone marrow, with a median frequency in bone marrow of 0.18% at baseline and 0.41% at week 18. This WT1 vaccination study provides immunologic, molecular, and preliminary evidence of potential clinical efficacy in AML patients, warranting further investigations.

Other approaches include the use of autologous DCs, generated from leukemia patients in CR and loaded with tumor antigens and/or the differentiation of leukaemia blasts into leukemic DCs. In particular, Van Tendeloo et al reported the results of a phase I/II clinical trial of WT1 vaccination based on antigen-loaded DCs with the induction of complete and molecular responses in some cases (Van Tendeloo et al, 2010). These results are promising. However, the clinical experience with DC-based vaccines targeting WT1 is far too limited and future studies are highly warranted to assess the role and the efficacy of these strategies of active immunotherapy in the clinical management of MRD in AML.

5. Conclusion

In conclusion, the clinical results of AML patients, especially if elderly, are particularly dismal, although the achievement of CR with MRD after combined chemotherapy appears as possible in the majority of patients. Unfortunately, the persistence of MRD leads to progression and patients ultimately die. For these reasons, alternative approaches for the prevention of relapse in CR patients are necessary and are currently under active investigation. In particular, the role of immunological therapies in the post-remission management of adult AML patients, such as NK therapy and active immunization against relevant tumor rejection antigens, including WT1, have been recently exploited with promising results in terms of immunological and clinical responses. Further studies (phase II-III) are highly warranted to really evaluate the role of such approaches and their impact on overall survival of AML patients.

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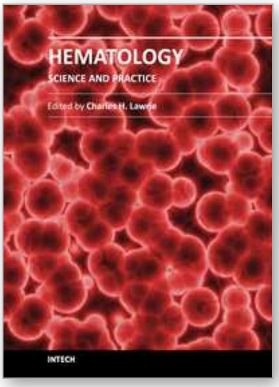

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