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**REVIEW**

# Immunotherapy with acute leukemia cells modified into antigen-presenting cells: *ex vivo* culture and gene transfer methods

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**Adult patients with acute leukemia have, in general, a poor prognosis, with long-term, disease-free survival achieved in only approximately one-third of cases. One of the proposed mechanisms for this poor overall response is the inability of the immune system to detect and eliminate residual malignant leukemia cells, which subsequently serve as a source of leukemic relapse. This review discusses the rationale of immunotherapy for acute leukemia and presents *in vitro* and *in vivo* model systems that were devised for pre-B acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML). New advances in the *ex vivo* manipulation of acute leukemia cells are presented, which attempt to modify these cells into functional antigen-presenting cells. These cells can then be used as autologous vaccines at the time of minimal residual disease after standard chemotherapy, to stimulate host immune responses against their own leukemia cells. The various approaches toward this aim include incubation of leukemia cells with cytokines or growth factors and gene manipulation of these cells. In particular, *ex vivo* culture of ALL cells with CD40 ligand, incubation of AML cells with granulocyte–macrophage colony-stimulating factor and interleukin-4 (GM-CSF/IL-4) and lentiviral transduction of ALL and AML cells for expression of immunomodulators (CD80 and GM-CSF) are current approaches under investigation for the development of autologous acute leukemia cell vaccines.**
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## Introduction

Conventional treatment modalities for patients with acute leukemia include chemotherapy, monoclonal antibodies and stem cell transplantation (SCT). Although hematologic remissions can be achieved in the majority of patients, long-term survival is modest at best, occurring only in 25–35% of adults with acute myeloid leukemia (AML)[1] and 30–40% of those with acute lymphocytic leukemia (ALL).[2] The major cause of relapse after chemotherapy is failure to have eradicated minimal residual disease, and these residual leukemia cells frequently show evidence of drug resistance. Consolidation of remission by means of high-dose chemotherapy with stem cell transplantation has failed to improve the outcome significantly.[3,4] The prognosis of acute leukemia is especially poor in the elderly, who frequently have unfavorable cytogenetic profiles and drug-resistant disease, and are often poor candidates for intensive therapies. Even in childhood leukemias, particularly ALL, prolonged survival may require use of therapies associated with severe, long-term complications. Thus, novel, more efficacious and less toxic treatments are clearly necessary.

Several facts support the concept that normal immune mechanisms can effectively target leukemia cells. After allogenic SCT for AML, freedom from relapse has been shown to correlate statistically with the presence of graft-versus-host disease, indicating that immune mechanisms are operative in inducing the graft-versus-leukemia (GVL) effect. The use of interleukin-2 (IL-2) has also been associated with induction and maintenance of remission in some patients with AML,[5,6] and reduction in the likelihood of relapse of ALL after autologous SCT.[7] While the anti-leukemia potential of immunotherapy has thus been demonstrated, it is clear that the GVL effect after allogenic SCT is weaker for AML than for chronic myelogenous leukemia (CML). This is further illustrated by the lower efficacy of donor lymphocyte infusions in patients with relapsed AML *vs* CML after SCT.[8]

The paucity of anti-tumor T cell-mediated immunity in the host with acute leukemia can be attributed to different factors. Important seems to be the inability of leukemia cells to function as competent antigen-presenting cells (APC), which has been correlated with their failure to provide sufficient costimulation to T cells.[9–11] This provides the rationale for attempting to modify leukemic cells into efficient antigen-presenting cells by *ex vivo* culture with immunomodulators, or by transfer of genes encoding cytokines or costimulatory molecules (Figures 1 and 2). This review summarizes the progress in this field and discusses the potential clinical applications of these approaches.

## Rationale: acute leukemia cells are intrinsically poor antigen-presenting cells

### *Requirements for T cell priming and activation*

An effective T cell-mediated immune response targeting leukemia cells requires that the patient's repertoire contain T cells that specifically recognize immunogenic 'tumor epitopes' presented by the malignant cells and/or by professional APC via cross-priming. For optimal activation and generation of productive T cell immunity, these T cells must receive two coordinated signals:[12,13] (1) *The cognitive signal*, which is provided to the T cell receptor (TCR) by major histocompatibility complex (MHC) molecules harboring a tumor antigenic epitope. This is an MHC-restricted signal and confers specificity of the response. MHC class I molecules trigger $CD8^+$ T cells and primarily present peptides derived from intracellular antigens,[14,15] although some APC can also present captured extracellular antigens in the MHC I pathway.[16] In contrast, MHC

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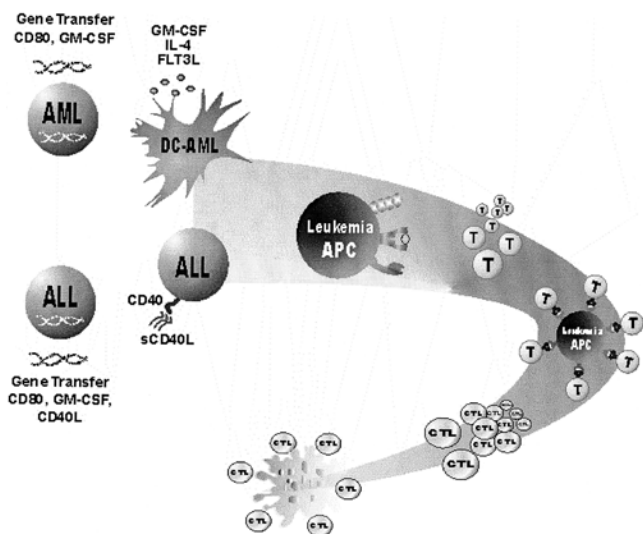


**Figure 1** Acute leukemia cells modified into antigen presenting cells by *ex vivo* culture or gene transfer methods. Acute myeloid leukemia (AML) cells and acute lymphocytic leukemia cells (ALL) can be genetically manipulated to express immunomodulators (eg CD80, GM-CSF) or treated with recombinant factors (eg CD40L, GM-CSF, IL-4, FLT3L) to direct their differentiation into 'leukemia-APC'. *In vitro* or *in vivo* stimulation of T helper and T cytotoxic precursor cells by the 'leukemia-APC', leads to the generation of anti-leukemia specific cytotoxic T lymphocytes (CTL).

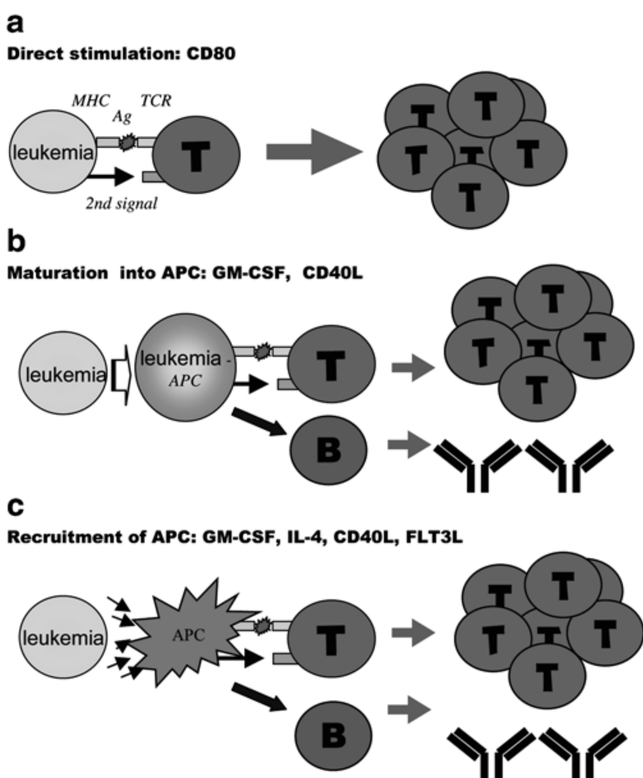


**Figure 2** Mechanisms proposed for the stimulation of immune responses through autologous acute leukemia cell vaccines genetically modified to express immunomodulators.

class II molecules trigger CD4$^+$ T cells, and present peptides localized in endosomal compartments, which primarily result from degradation of extracellular antigens.[14,17] (2) *The costimulatory signal*, which is delivered to appropriate receptors on T cells by their cognate ligands expressed on the APC. This signal is neither MHC-restricted nor antigen-specific. Costimulation is critical to IL-2 production and T cell clonal expansion.[18] Various mechanisms of T cell costimulation have been proposed, involving different ligand–receptor pairs whose coordinate engagement with a TCR signal ultimately leads to cytokine production and mediates T cell proliferation.[19,20] The major costimulatory pathway involves the interaction of the B7 family ligands (CD80/B7-1 and CD86/B7-2) expressed by the APC, with the CD28 receptor expressed on the T cell surface.[21]

Although this model of T cell activation might be an oversimplification of a more complex and dynamic process,[20] the requirement of the engagement of a specific and functional TCR repertoire is clear, and the role of costimulation is well supported by a wide body of evidence, including observations using knock-out mice.[22]

## *'Professional' antigen-presenting cells*

'Professional' APCs are cells specialized in the processing and presentation of both exogenous and endogenous antigens. These cells play a major role in the continuous scrutiny of the T cell repertoire, ensuring that suitable T cell immunity is triggered when needed. Dendritic cells (DCs) and B cells serve as 'professional' APCs, and both derive from multipotent hematopoietic stem cells (HSC). Common attributes of 'professional' APCs are their constitutively high expression of MHC molecules, and the expression, either constitutive or inducible, of costimulatory molecules.

DCs are the most potent 'professional' APCs in the immune system, with multiple characteristics contributing to this capability, including: (1) the expression of receptors for antigen uptake that allow an efficient capture of exogenous antigens; (2) a molecular machinery of antigen degradation and processing for both exogenous and endogenous molecules; (3) the efficient transport, assembly and presentation of antigens by highly expressed MHC I and MHC II molecules; (4) the expression of adhesion and costimulatory molecules, such as CD80, CD86, CD54 and CD58; (5) the secretion of pro-inflammatory immunomodulators, such as interferon-gamma (IFN-$\gamma$), interleukin-12 (IL-12) and interleukin-15 (IL-15); (6) their strategic distribution in the body at locations optimal to screen and capture antigens; and (7) their exquisite ability to migrate to lymphoid organs, where captured MHC-bound epitopes can be presented to the scrutiny of the T cell repertoire.[23,24] From these characteristics it can be logically assumed that the 'differentiation' of leukemia cells into dendritic cells could represent an effective approach to augmenting the immunogenicity of these malignant cells.

It has been demonstrated that DCs can differentiate from the hematopoietic myeloid progenitor (myeloid DCs) or from lymphoid-committed progenitor cells (plasmacytoid DCs). An updated description of DC differentiation is reviewed elsewhere.[25,26] An important characteristic of DCs is the considerable changes in their capability to process antigens and to stimulate T cells, based upon their degree of differentiation. Immature DCs are very efficient in capturing and processing antigens, but poor in stimulating T cells, whereas mature DC are powerful mediators of T cell priming and activation, but

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inefficient at antigen processing. These factors are relevant, as leukemia cells differentiated into DC-like cells should be capable of processing and presenting leukemia-associated antigens and, upon appropriate maturation and activation, should have the capacity to trigger an efficient T cell-mediated response.

It is well established that mature B cells are capable of capturing, processing and presenting antigens in the context of their abundant MHC molecules. B cells also express costimulatory molecules, particularly following adequate stimulation. It remains debatable whether B cells play any physiological role as professional APC in T cell-mediated immunity,[27] although it has been demonstrated that activated mature B cells can be employed to generate and amplify epitope-specific T cell responses to tumor antigens.[28] B cells derive from lymphoid progenitors and develop along discrete stages of differentiation, proliferation, selection and maturation that take place initially in the bone marrow and later in various lymphoid tissues (human B cell development is reviewed elsewhere[29,30]). Little is known about the immunogenicity of normal B-lineage cells along their differentiation pathway, in terms of the priming and stimulation of T cells.

### *Acute leukemia cells as antigen-presenting cells*

Acute leukemia cells originate from the hematopoietic progenitor, and represent the malignant counterpart of hematopoietic cells transformed at different stages of their differentiation pathways. A consequence of the malignant transformation is the failure of their terminal maturation and the functional impairment of these cells. The leukemic blasts are generally believed to have limited proliferative capacity, suggesting that a small subpopulation of leukemic stem cells that possess extensive proliferative capacity and potential of self-renewal must maintain the malignant clone.[31] The understanding of the processes that mediate and regulate the differentiation pathways of these malignant cells should provide valuable insights into the strategies that may be employed to 'differentiate' acute leukemia cells into competent and powerful APCs.

Acute leukemia cells are generally poor APCs and fail to induce significant T cell proliferation even in the allogeneic setting,[9,32,33] For most patients, the acute leukemia blasts express both MHC I and II molecules for antigenic presentation,[9,10,34,35] thereby discounting MHC expression as the limiting factor. Moreover, leukemia cells can induce T cell alloreactivity if a signal mimicking costimulation is provided, thus demonstrating that the MHC expression by leukemia cells is sufficient and intact.[9] Importantly, the expression of costimulatory ligands by acute leukemia cells is heterogeneous, but usually insufficient or lacking. It has been shown that most primary acute leukemic cells do not express the costimulatory ligand CD80 (<10%), and a variable percentage of CD86 expression has been documented (20–50%).[9,10,34] The critical role of B7-family members in anti-leukemic T cell responses has been demonstrated in multiple studies in which the costimulatory signals have been introduced by either leukemia cell stimulation or gene transduction, or delivered by stimulatory antibodies. Finally, the induction of T cell anergy and escape from productive immunity induced by 'tolerogenic' leukemia cells or by cross-presentation of tumor antigens by APC may play a role in the development of these leukemias.[9,36]

## Acute leukemia cell differentiation into competent antigen-presenting cells

### *B cell precursor acute lymphocytic leukemia*

Several strategies for immunotherapy of ALL were designed primarily to stimulate, in a non-specific manner, the patient's immune cells, or to increase the sensitivity of leukemia cells to immune responses.[37] Examples of such approaches are the use of exogenous cytokines, mainly IL-2 and interferons, but also interleukin-4 (IL-4), interleukin-7 (IL-7), IL-12 and granulocyte colony-stimulating factor (G-CSF). Multiple *in vitro* studies, animal models and clinical trials have been performed with variable results and, generally, without striking success. Another strategy has been the use of a combination of bispecific CD3 × CD19 and CD28 bivalent antibodies to stimulate T cell responses. This approach allowed the mobilization of both $CD4^+$ and $CD8^+$ T cells and increased the CTL response directed to autologous blasts.[38] However, a potential limitation of these strategies, well documented in studies using IL-2, is their lack of specificity for leukemia.

Another approach aims at mobilizing the patient's antitumor immunity via the recruitment and amplification of leukemia-specific responses using competent APC. Here, either leukemia cells are modified to become immunogenic APCs, or the patient's DCs are activated to trigger anti-leukemia T cell responses.[32,39] The use of the 'CD40 ligand system' has been shown to be a valuable strategy to improve leukemia cell immunogenicity (Figure 1). This strategy exploits the physiological role of CD40 ligand (CD40L) as an important modulator of B cell biology.[40] The cross linking by CD40L of its cognate receptor CD40, expressed by both normal and malignant B-lineage cells, induces or up-regulates the expression of multiple molecules that play important roles in T cell responses. In B cell ALL, CD40L dramatically improves the immunogenicity of leukemia cells, which become competent APC in both autologous and allogeneic settings.[9,32] The ligation of CD40 on ALL cells results in the up-regulation of MHC I and MHC II, the induction of CD80/B7-1 and the induction or up-regulation of CD86/B7-2.[9] Importantly, this strategy has been employed in an attempt to generate patient T cells that are able to lyse autologous leukemia cells in an efficient and specific manner.[32] These anti-leukemia CTL were able to migrate through vascular and bone marrow endothelium and lyse the leukemia blasts even in the presence of a leukemia-permissive bone marrow stroma.[41] More recently, we have observed that CD40L also induces leukemia cells to produce the CC-chemokines MDC and TARC, which mediate the transendothelial migration of anti-leukemia CTL.[42] These data constituted the foundation for an on-going clinical trial (Dana-Farber Cancer Institute, Boston, MA, USA) using irradiated CD40-stimulated leukemia cells as therapeutic vaccines.

### *Acute myeloid leukemia*

As for ALL, cytokines have been used in patients with AML, aimed at the mobilization and amplification of an effective immune response. Several cytokines, such as IL-2, interferons, granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), interleukin-6 (IL-6), and FLT3 ligand (FLT3L), have been employed in different experimental models and in clinical protocols.[5,43–45]

A strategy that has been extensively pursued in AML is the

differentiation or maturation of leukemia cells into DCs (Figure 1). This approach is the logical reflection of the knowledge of DC development from myeloid primitive cells. Different strategies, employing various cocktails of cytokines (such as GM-CSF, IL-4, interleukin-13 (IL-13), tumor necrosis factor alpha (TNF-$\alpha$), FLT3L) and immunomodulators (as soluble CD40L) have been employed. Although different protocols have been employed, they generally are based on a 'DC-inducing cocktail' containing GM-CSF and IL-4, which has been identified as essential for the generation of DC from blood monocytes.[46] In a representative study, Choudhury and colleagues[44] demonstrated that GM-CSF + IL-4 in combination with CD40L or TNF-$\alpha$ could induce the differentiation of 18/19 primary AML samples into cells with the morphology, phenotypic characteristics and T cell stimulatory properties of DC. Importantly, autologous T-lymphocytes co-cultured with AML-DC were able to efficiently lyse autologous leukemia targets, but not normal autologous cells obtained during remission. This observation was confirmed by several other studies, which aimed at defining optimal conditions to generate AML-DC by addition of other cytokines, such as stem cell factor (SCF),[43,47] TGF-$\beta$1,[45,47] or FLT3-L.[43] Furthermore, culture of primary AML cells with GM-CSF, IL-3 and IL-6 promoted extended cell viability and resulted in variable up-regulation of CD80, which stimulated a more vigorous allogeneic and autologous anti-leukemia response.[48] Since AML is a heterogeneous disease, further studies are required to predict the potential of leukemic cells from individual patients to differentiate towards a DC phenotype. As culture conditions are optimized for the different subtypes of AML, the full impact of AML-DC will be ascertained.

## Direct gene transfer to improve immunogenicity of leukemia cells

### *Gene transfer to combine different pathways of immune stimulation*

The direct gene transfer of immunomodulators into acute leukemia cells as a mean to improve their immunogenicity is an alternative approach to the treatment of leukemia cells with exogenous factors (Figures 1 and 2). The rationale for this strategy is: (1) ALL and AML cells show variable responses to *ex vivo* stimulation with exogenous immunomodulators, which often fail to significantly improve their immunogenicity. (2) Leukemia cell vaccines transduced to stably express immunomodulators would have a more enduring immunization effect due to a more persistent presence of these molecules. (3) Combinatorial gene transfer could promote synergistic effects through activation of different pathways of immune-stimulation (Figure 2): (a) expression of co-stimulatory ligands (eg CD80, CD86) that facilitate T cell priming; (b) expression of factors that trigger inflammation (eg GM-CSF, IL-2, chemokines) and recruit high numbers of professional APC to the vaccination site; (c) expression of immunomodulators (eg GM-CSF, CD40L) that promote the differentiation/maturation of leukemia cells into effective APC.

### *Murine models for genetically modified acute leukemia*

The direct transfer of genes into leukemia cells that enhance their APC function has been evaluated in several pre-clinical murine models. We have evaluated the stimulation of immune responses specifically directed to aggressive Philadelphia chromosome-positive ALL in the BM185/Balb/c transplantable syngeneic murine model.[49] The BM185wt pre-B cell line is derived from BALB/c bone marrow genetically modified to express the p185 BCR-ABL oncogene, which causes leukemic transformation.[50] Immunocompetent syngeneic BALB/c mice injected with as few as $10^3$ BM185 cells develop leukemia, with death within 3 weeks of the intravenous challenge.[49] Mice in advanced stages of disease develop high white blood counts, and the presence of BCR-ABL transcripts in peripheral blood samples is detectable by RT-PCR at early stages of disease (7–10 days post challenge). Therefore, BCR-ABL or other antigens up-regulated by the leukemogenic event could provide leukemia-associated antigenic determinants to be explored in the setting of genetically modified autologous Ph+ ALL cell vaccines.

The *in vivo* effects of the expression of IL-2, GM-CSF and CD80 by leukemia cells transduced with Moloney leukemia virus (MLV) vectors have also been examined in this model.[49] Clonal leukemia cell lines expressing high amounts of the immunomodulators were selected. The expression of CD80 by challenged live leukemia cells resulted in significant decrease of leukemia incidence, whereas the expression of IL-2 and GM-CSF promoted only weak effects in disease development. BM185 cells expressing CD80 were rejected in 50% of the mice challenged at a cell dose ($5 \times 10^4$) that causes 100% lethality for the parental BM185 leukemia cells.[49] Importantly, long-term survivors to the BM185/CD80 challenge remained PCR negative for the BCR-ABL oncogene for at least 6 months and without noticeable signs of auto-immunity. *In vivo* immunodepletion experiments showed that both $CD4^+$ and $CD8^+$ T lymphocytes mediated the anti-BM185 immune responses triggered by CD80-BM185 cells and conferred protective immunity to subsequent challenges with the parental leukemia. CTL responses after BM185/CD80 challenge were shown to be vigorous and directed against the parental BM185 leukemia, with little or no reactivity against other syngeneic cells.

To more accurately reproduce the clinical setting, experiments were designed to evaluate the prophylactic and therapeutic efficacy of the BM185/CD80 irradiated cell vaccines. Despite the strong stimulation mediated by live BM185/CD80 cells, only a modest protection with irradiated cell vaccines was observed. These studies demonstrated that vaccination after established disease inhibited leukemia growth, thus delaying disease progression, but did not confer long-term leukemia-free survival.

In subsequent studies, the combined expression of CD80 and a soluble immunomodulator (IL-2 or GM-CSF) were evaluated in terms of their ability to augment the anti-leukemia immune response and cure pre-established leukemia in the BM185 murine model.[51] BM185 cells co-expressing CD80 and GM-CSF were consistently more effectively rejected than cells expressing either molecule alone. Interestingly, irradiated BM185/CD80/GM-CSF cell vaccines promoted the highest CTL response against wild-type BM185,[51] which correlated with a higher protective immunity against subsequent leukemia challenge (80% survival). Growth of BM185 leukemia cells implanted subcutaneously was drastically reduced with subsequent distal vaccination with BM185/CD80/GM-CSF-irradiated cells. Histopathological examination of the vaccination site showed a significant infiltration of $CD4^+$ and $CD8^+$ T cells, as well as dendritic cells. When the leukemia cells were transplanted intravenously prior to vaccination, a rapid

systemic dissemination of the leukemia occurred, preventing any therapeutic benefit due to the vaccination alone. Therefore, active immunotherapy for rapidly progressive disease will require additional means to slow the initial leukemic proliferation, by means of cytotoxic therapy.

For acute myeloid leukemia, the most extensively studied model consists of radiation-induced AML cells that are maintained by serial passages in syngeneic SJL/J mice. Spleen mononuclear cells (mainly myeloblasts) have been isolated from mice presenting with advanced disease and were genetically modified with ecotropic MLV vectors to express immunomodulators.[52] Administration of AML cells transduced to express CD80 resulted in $CD8^+$ T cell-dependent rejection of leukemia in the mice vaccinated at early stages of disease.[52] Early use of chemotherapy followed by vaccination with CD80 expressing AML cells cured 100% of the leukemic mice.[53] In a further report, the co-expression of cytokines GM-CSF, IL-4, TNF-$\alpha$ and the costimulatory ligand CD86 were evaluated for their potential to improve the vaccination potential of AML cells.[54] All transduced immunomodulators promoted some degree of leukemia rejection or delay when expressed by live AML cells, although only the vaccines expressing GM-CSF, but not CD86, IL-4 or TNF-$\alpha$, could elicit anti-leukemia-specific immunity.[54] Vaccination with irradiated AML cells expressing GM-CSF provided better protection against pre-established leukemia than cells expressing CD80.[54] More recently, this model has been used to evaluate AML vaccines expressing IL-12, an APC-secreted cytokine that mediates the stimulation of cytotoxicity and proliferation of NK and CTL, the induction of cytokine and chemokine secretion, and the generation of T helper type 1 cells (Th1). AML/IL-12 irradiated vaccines could cure mice bearing advanced disease without signs of systemic IL-12 toxicity.[55] The cell vaccination approach, which triggered an anti-leukemic CTL response, was more effective than the systemic administration of IL-12, which delayed leukemia progression, but was incapable of promoting leukemia rejection.[55]

In another murine AML model, non-leukemic 32DC13 cells were transformed by transfection of the BCR-ABL gene generating a subline that was leukemic and lethal to syngeneic, immunocompetent C3H/HeJ mice. Live AML cells genetically modified to express CD80, but not CD86, were very effective as live vaccines in inducing protective and therapeutic immunity against challenge with the parental leukemia.[56,57] Irradiation of $CD80^+$ transduced cells abrogated the protective immunity, in accordance with another AML model using CD80-transduced WEHI3B myelomonocytic leukemia cells.[58] The simpler explanation for the lack of immunogenicity of irradiated CD80-expressing leukemia vaccines is that these cells failed *in vivo* to recruit the repertoire of leukemia-reactive T cells, likely due to the poor viability of the vaccines. Supporting this hypothesis, it was shown in the M1 myelocytic leukemia model (syngeneic to SL mice) that a single vaccination with M1/CD80 cells provided only a minor protection against leukemia, which could be improved by employing multiple M1/CD80 vaccinations.[59] Taken together, these studies demonstrated that irradiation abrogates some of the immune effects of CD80-expressing leukemia myeloid vaccines, which can be overcome by repeated cycles of vaccination.

In the WEHI3B and EL-4 models, irradiated GM-CSF-transduced leukemic cell vaccines also provided strong immunoprotection against leukemia.[58] Importantly, a significant synergistic effect was obtained by the combined administration of irradiated EL-4 cells singly transduced to express CD80 or GM-CSF.[58] The combination of CD80 with other immunomodulators in a cell vaccine was studied in an AML murine model using the DA1-3B leukemia cell line genetically modified to express BCR-ABL. Injection of $10^4$ leukemia cells into CH3 mice resulted in death after approximately 30 days. DA1-3b cells were stably transfected with plasmids encoding CD80, GM-CSF and/or CD40L and their potential as vaccines were compared. Co-expression of CD80 and GM-CSF in a cell vaccine conferred the best protective and therapeutic potential, whereas the expression of CD40L alone was the next most effective.[60]

Although other immunomodulators and hematopoietic regulators, alone and in combinations, remain to be evaluated, the pre-clinical data accumulated, by ourselves and others, support the following assumptions: (1)The expression of CD80 or GM-CSF in acute leukemia cells increases their immunogenicity. (2) Irradiated $CD80^+$ leukemia cell vaccines confer T cell-dependent protective immunity, while responses to irradiated GM-$CSF^+$ leukemia cell vaccines are mediated by both T cells and professional APC. (3) The effects of the two immunization strategies can be combined to potentiate an optimal therapeutic effect. (4) Vaccination is most likely to be effective in the early stages of disease and use as an adjuvant therapy to control minimal residual disease is most likely to confer more favorable results. (5) Although it is conceivable that antigens presented by the genetically modified leukemia cell vaccines are also expressed by normal hematopoietic cells, none of the published studies have noted the development of undesirable side-effects in the vaccinated mice, such as bone marrow aplasia or peripheral cytopenia. Thus, no toxicity related to auto-immunity is anticipated as a result of leukemia cell vaccination.

Taken together, results from pre-clinical murine models indicate that acute leukemia cell vaccines co-expressing CD80 and GM-CSF are effective and safe and merit clinical evaluation.

## *Gene transfer into primary acute leukemia cells*

As cell vaccines move towards clinical application, an increasing effort has been placed in the development and evaluation of genetic vectors for the efficient transduction of primary leukemia cells. Different types of gene transfer approaches have been tested in primary human leukemia cells, including those based on murine leukemia viruses (MLV),[61–63] Herpes viruses,[64] and plasmid transfection.[61,63,65] These methods either lacked the necessary consistency or failed to successfully confer high rates of gene transfer at low cytotoxicity.

Efforts to employ adenoviruses in leukemia cell transduction have likewise been ineffective.[63,66,67] Methods have been devised to improve adenoviral-mediated gene delivery into AML cells, including the treatment of leukemia blasts with a DC-inducing cocktail of growth factors (GM-CSF, IL-4 and CD40L).[63] Another approach has been the generation of adenovirus–polycation complexes that may partially overcome the lack of the adenovirus cognate CAR receptor on hematopoietic cells.[68] However, despite their broad ongoing clinical use, adenoviral vectors are highly immunogenic *per se*[69,70] and the presentation of dominant viral epitopes will likely mask weaker leukemia-associated antigenic determinants, therefore potentially diverting the host immunity towards an anti-vector rather than an anti-leukemia-specific response.

Due to the poor and/or inconsistent gene transfer obtained

with the above vectors, the human immunodeficiency virus (HIV-1)-derived lentiviral vectors were investigated. Lentiviruses are complex retroviruses that in nature infect macrophages and lymphocytes and are effective in infecting nonproliferating or growth arrested cells *in vitro*, including hematopoietic stem cells.[71,72] The transgene incorporates stably into the genome and there is no cytotoxic effect upon transduction. One possible mechanism leading to infection of non-replicating cells is the presence of nuclear localization signals in the lentiviral pre-integration complex that mediates its active transport through the nucleopores during interphase.[73,74] Primary leukemia cells show poor proliferation *in vitro*;[75] therefore, lentiviral vector transduction could be a potentially valuable alternative for gene transfer. This hypothesis was validated by comparing gene delivery with MLV (which requires cell replication for infection) and HIV-derived lentiviral vectors. We observed that only lentiviral vectors transferred the green fluorescent protein (GFP) reporter gene at high frequencies in primary ALL cells.[61]

The lentiviral vector system for the delivery and stable expression of the CD80 and GM-CSF genes in human leukemia cells was subsequently chosen for further clinical development.[76] VSV-G pseudotyped lentiviral vectors expressing CD80 and GM-CSF alone or in combination were efficiently produced by transient co-transfection of 293T cells with second generation lentiviral vectors[76,77] and concentrated at high titers ($10^8$–$10^9$ infective particles/ml). Expression of CD80 was stable for at least 2 weeks and was detected at high levels in transduced human ALL, AML and CML lines (75–95% $CD80^+$ cells) and cryopreserved primary human AML and ALL cells (40–95% $CD80^+$ cells). GM-CSF expression and secretion by the leukemia cells was also documented.[76]

Functional experiments were then performed to evaluate the response of the patients' autologous T cells against autologous CD80-transduced leukemia cells. The stimulatory activity of non-transduced and transduced ALL cells was compared in primary mixed lymphocyte reactions (MLR). The primary MLR showed that ALL/CD80 cells, but not ALL/Mock or ALL/GFP, stimulated significant T cell proliferation. T cells primed during the primary MLR cultures were rested and then rechallenged, in a secondary MLR, with ALL/Mock, ALL/GFP or ALL/CD80 cells. T cells primed with ALL/CD80 proliferated when rechallenged with ALL/Mock or with ALL/GFP, thus demonstrating that, in a secondary MLR, primed T cells can recognize and respond to leukemia cells that are inefficient APCs. Importantly, the primed T cells showed a significantly more robust proliferative response when rechallenged with ALL/CD80 cells. Furthermore, only T cells primed with ALL/CD80 cells could be used for the rechallenge experiments as the co-culture of T cells with ALL/Mock or ALL/GFP cells failed to stimulate significant autologous T cell proliferation in the primary MLR.[76] Primary MLR in the presence of an anti-CD80 blocking antibody or the fusion protein CTLA4-Ig, which blocks the engagement of CD28 by CD80, resulted in a dramatic inhibition of the autologous T cell proliferation promoted by $CD80^+$ ALL cells.[76] These results demonstrated that the transduction of CD80 into ALL cells was necessary and sufficient to modify the leukemia cells into competent APC.

We have recently evaluated third generation self-inactivating (SIN) lentiviral vectors, which have the potential advantage of improved safety.[78] We observed that SIN lentiviral vectors efficiently transduced AML cells from bone marrow and peripheral blood diagnostic samples obtained from adult patients, as analyzed by flow cytometry and ELISA at 48 h post-transduction. Long-term effects of gene delivery were observed in some of the primary AML samples transduced with the vector RRL-GM-CSF and the bicistronic vector RRL-GM-CSF/CD80, as demonstrated by the dendritic cell phenotype, including larger cell volume, dendrites and pseudopods, and the expression of CD80, CD86 and CD1a.[78] Cell proliferation and viability, assessed by $^3$H-thymidine incorporation and by MTS assays, respectively, were both stimulated by transduction with the RRL-GM-CSF vector compared with the RRL-GFP control.[78] MLR using irradiated (3200 cGy) transduced AML cells, showed that transduction with RRL-CD80, RRL-GM-CSF and RRL-GM-CSF/CD80 significantly increased allogeneic T cell proliferation and provided a modest increase in autologous T cell proliferation. Analysis of CD69 expression, a marker for T cell activation, showed that activation of $CD4^+$ cells was enhanced by co-culture with allo-AML/RRL-GM-CSF cells, whereas $CD8^+$ activation was superior for co-cultures with allo-AML/RRL-GM-CSF and allo-AML/RRL-GM-CSF/CD80 cells. Expression of GM-CSF and/or CD80 by autologous stimulatory AML cells correlated with increase in the percentage of $CD4^+/CD69^+$ cells and some decrease in $CD8^+/CD69^+$ cells, indicating a shift towards the T helper response.[78] We are currently evaluating the effect of the expression of combined transgenes (GM-CSF, IL-4, FLT3L, CD40L) in triggering the differentiation of primary AML cells into mature APCs. We were able to show, in co-transduction experiments, that up to four different genes can simultaneously be expressed in AML cells (Stripecke *et al*, manuscript in preparation).

In summary, these studies showed the feasibility of the transduction of primary leukemia cells with lentiviral vectors expressing different immunomodulators, which can be combined to elicit complementary pathways of anti-leukemia immune responses. The advantages of lentiviral vectors for development of autologous leukemia cell vaccines include: (1) consistent gene-delivery efficiency; (2) high levels of transgene expression; (3) persistent expression of the transduced gene; (4) no viral proteins as only the transduced gene is expressed; (5) no undesirable cytotoxic effects; and (6) simplicity of use (leukemia cells are exposed to virus only once).

## Considerations for clinical application of designed acute leukemia cell vaccines

### *Therapy of minimal residual disease*

The state of minimal residual disease (MRD) achieved either after conventional chemotherapy or SCT is an ideal setting for trials of leukemia cell vaccines. By definition, these patients generally have a low burden of malignant disease and therefore, immune intervention is likely to be more effective provided that the patient's immunity is not hampered. The use of targeted chemotherapy (eg anti CD33-calicheamycin conjugate for AML[79]) would be ideal to achieve initial debulking of disease, especially if systemic immunosuppression could be avoided. Leukemia cells can be harvested at diagnosis by bone marrow aspiration or leukopharesis and cryopreserved. Following achievement of remission, these cells can be modified *ex vivo* into APCs as described above, irradiated and injected as a vaccine. The optimum timing of administration of these vaccines after initial chemotherapy will require investigation. These vaccines can also be potentially combined with Th1-inducing cytokines, such as IL-2 or IL-12 to further augment immune responses. Although freedom from relapse

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would be the ultimate test of the efficacy of these vaccines, anti-leukemia immune responses can also be monitored by assays like ELISPOT. What remains largely unknown, yet is critical for the use of such strategy, is: (1) the magnitude and duration of 'immunodeficiency' caused by conventional cytotoxic therapy; and (2) whether anti-leukemia immunity remains intact after chemotherapy.

### *Therapy of refractory/relapsed disease*

Immunotherapy may possibly be able to induce remissions in patients with active disease as shown by the success of delayed lymphocyte infusion (DLI) or high-dose IL-2 in the setting of relapse after allogeneic SCT for patients with CML and AML. The use of leukemia cell vaccines as discussed above, in combination with high-dose cytokines, such as IL-2 can be considered. Another potential application of leukemia cells modified into efficient APC would be their use in *ex vivo* expansion of anti-leukemia-reactive T cells for adoptive immunotherapy. This concept has been demonstrated in murine models by the delivery of the CD80 gene into AML cells. The transduced cells were used to stimulate proliferation of anti-leukemia reactive T cells, which were then administered to syngeneic or allogeneic recipients to stimulate GVL.[80,81]

Generation of CTL clones or lines using leukemic APC is another feasible approach. Minor histocompatibility antigen-specific CTL clones can be generated from donors of allogenic SCTs using modified leukemia/APC cells. In a xenogeneic model of human AML, such clones could inhibit the engraftment of leukemia stem cells in NOD/SCID mice.[82] In another approach, it was shown that low levels of CD80 gene transfection into human AML cells induced primary T cell responses from an MHC-identical bone marrow donor in mixed lymphocyte cultures, allowing the generation of leukemia-reactive CD4$^+$ T cell lines and clones, which in 10/35 cases were leukemia-specific.[65] Therefore, lentiviral CD80 gene delivery offers a promising method to produce primary autologous or allogeneic T cell responses directed at the patient's leukemia-associated antigens and/or minor histocompatibility antigens. These T cell clones would be expected to be more efficient than DLI and less likely to induce graft-versus-host disease.

### *Safety of lentiviral vectors*

Biosafety issues must be considered prior to the clinical application of lentiviral vectors, including the occurrence of replication-competent lentivirus (RCL) and vector mobilization. Due to the predicted short lifespan of the irradiated acute leukemia cells (2–5 days), the probability of recombination over time is reduced. Because the consequences of clinical gene transfer with HIV-derived lentiviral vectors are not yet foreseeable, considerable effort has been invested in the generation of more efficient and safer lentiviral vectors.[77,83–85] In 'second generation' lentiviral vectors, four HIV genes (*vif*, *vpr*, *vpu* and *nef*) critical for pathogenesis are absent in the viral vector.[83] More recently, it has been shown that the *tat* gene is also dispensable,[84] and a self-inactivating (SIN) lentivirus vector has been designed in which a 400-nucleotide deletion in the 3′ long terminal repeat (LTR) abolishes the LTR promoter activity and hampers recombination with wild-type HIV in a hypothetical infected host.[83] The SIN vectors with a tat-independent promoter have been termed 'third generation' lentiviral vectors. These safer, self-inactivating lentiviral vectors expressing immunomodulators were recently evaluated for the development of acute leukemia cell vaccine preparations.[78] Various groups are currently examining the large-scale production, testing and certification of these lentiviral vectors for clinical use.

We propose to develop a phase I dose escalation vaccination trial using intradermal injections of irradiated autologous leukemia cells transduced with self-inactivating lentiviral vectors. In this first clinical trial, adult AML patients in clinical remission after chemotherapy, but with high risk of relapse will be enrolled. Based on our successful pre-clinical studies in both murine models and *in vitro* assays using human primary leukemia cells, this trial has been designed to evaluate the immunotherapeutic potential of an AML vaccine transduced with the combination of GM-CSF and CD80.

## Perspectives and conclusions

Immunotherapeutic approaches for acute leukemia have largely been limited to trials of cytokine therapy. Peptide vaccination is another feasible approach, but requires knowledge of the antigenic determinants and their HLA restriction. To date, only a few leukemia-associated tumor antigens have been identified. These antigens include the TEL-AML1 fusion gene, the tumor suppressor WT1, and the minor histocompatibility antigens HA-1 and HB-1.[86–90] Important questions remain to be resolved, namely: (1) the representation of T cells reactive to each of these epitopes in the patient's T cell repertoire; (2) the potency of the cytotoxic responses *in vivo*; and (3) the specificity of such responses. In our own studies, we have observed that in HLA-A*0201$^+$TEL-AML1$^+$ALL patients, the T cell response directed to the TEL-AML1-derived epitope represented only a small fraction of the overall anti-leukemia CTL reactivity (Cardoso *et al*, manuscript in preparation). A potentially important limitation is that these epitopes can only be employed in patients expressing these antigens (TEL-AML1 in about 25% of B-ALL patients; HA-1 in about 69% of the population) and expressing the proper MHC I molecule (HLA-A*0201 present in 30–50% of Caucasians). Therefore, the clinical usefulness of these leukemia-associated antigens remains to be determined. In contrast, leukemia cells modified into competent APC would likely result in the presentation of multiple leukemia-associated antigens without requiring knowledge of the identity of these antigens.

All together, immunotherapy with acute leukemia cell vaccines is still in its infancy. We have learned which approaches may be likely to stimulate potent and specific anti-leukemia immune responses from *in vitro* and *in vivo* experimental models. It is now nearing the time to translate these approaches to the clinical arena in an attempt to understand and optimize host immune responses against leukemia with the ultimate goal of eradicating minimal residual disease.

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