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SYMPOSIUM PAPER

Development of a dendritic cell-based vaccine for chronic lymphocytic leukemia

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Abstract Evidence for the existence of CLL-specific antigens recognized by the immune system can be gathered from the observation that many patients display monoclonal or oligoclonal expansions and skewed repertoire of T cells. In vitro functional studies have shown that tumor-specific T-cells are able to lyse the leukemic cells. Antileukemic cellular immunity may be boosted in vivo using dendritic cell-based immunotherapy. Our preclinical studies provide evidence that DC that had endocytosed apoptotic CLL cells (Apo-DC) were superior to fusion hybrids, tumor lysate or RNA in eliciting antileukemic T-cell responses in vitro. We have validated a method for enriching the small number of monocyte precursors present in the peripheral blood of CLL patients and utilize them for generating individualized, Apo-DC cellular vaccines. In most cases, a minimum of 50×10^6 Apo-DC could be generated, beginning with immunomagnetically enriched monocytes from a single leukapheresis product containing at least 1% CD14+ cells. Cryopreservation and thawing did not affect the phenotype or the T cell stimulatory function of Apo-DC. A phase I/II, open label clinical trial examining the feasibility, safety and immunogenicity of Apo-DC vaccination has been initiated. CLL patients receive 10^7 Apo-DC for at least five immunizations and monitored clinically and immunologically for 52 weeks. Three cohorts are accrued stepwise. Cohort I receives Apo-DC alone; Cohort II: Apo-DC+ repeated doses of low-dose GM-CSF; Cohort III: low-dose cyclophosphamide followed by Apo-DC + GM-CSF.

Keywords CLL · Dendritic cell · T cell · Immunotherapy · Clinical trial

Introduction

Chronic lymphocytic leukemia is a malignant lymphoproliferative disorder characterized by the progressive accumulation of monoclonal CD5+/CD23+ B lymphocytes in the blood, bone marrow and lymphoid organs. The clinical course of chronic lymphocytic leukemia (CLL) is heterogeneous. The majority of the patients remain stable for a long time without need for therapy, while others progress rapidly and succumb to treatment-refractory disease. There has been a remarkable progress in the treatment of CLL but the disease is still considered incurable. Chemotherapeutic agents, such as purine analogs and alkylating agents, and monoclonal antibodies, alone or in combination, succeed in achieving high complete response rates and prolonging progression-free survival, but do not yet have a significant impact on overall survival [6, 13]. Additionally, chemo-immunotherapy regimens that presently demonstrate the greatest clinical efficacy are typically associated with protracted decline in cell mediated immunity and increased risk of infections and may be less well tolerated by elderly patients. Allogeneic stem cell transplantation is potentially curative, but is encumbered by treatment-related toxicity and paucity of HLA-matched donors.

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There are several considerations that make antileukemic vaccination a potential therapeutic approach for CLL. Firstly, the majority of CLL patients have advanced age and need treatment options with low toxicity. Secondly, most of the patients have a long period of indolent disease which does not need aggressive intervention. Thirdly, the utility of an antileukemic immune response in controlling the disease is emphasized by the graft-versus-leukemia effect associated with allogeneic stem cell transplants and donor lymphocyte infusions.

In this article, we review our preclinical studies which form the basis for the development of a cellular vaccine for the treatment of CLL. The clinical and immunological efficacy of this vaccine is being investigated in an ongoing phase I–II clinical trial.

CLL-specific T-cell responses

Anecdotal, clinical observations point toward the existence of spontaneous immunity against tumor cells and the possibility to potentiate such immune responses by immunotherapy. Disease remissions concomitant to increased immune activity due to viral infections have been described [34]. Clinical responses following treatment with immunomodulatory cytokines [44] and a graft-versus-leukemia effect after allogeneic hematopoietic cell transplantation (HCT) or donor lymphocyte infusions (DLI) are compelling manifestations of the therapeutic potential of immune responses [12, 15, 26, 28].

A number of TAA have been identified in CLL and in many instances functional studies have been performed to verify the existence of naturally occurring, reactive T cells. Examples are fibromodulin [30, 31], the receptor for hyaluronic acid mediated motility (RHAMM/CD168) [14], murine double-minute 2 oncoprotein (MDM2) [29], telomerase reverse transcriptase (hTERT) [7], the oncofetal antigen-immature laminin receptor protein (OFAiLRP) [37] and the tumor-derived Ig VH-CDR3 region [18, 39]. The latter is specifically expressed by the tumor clone as surface membrane immunoglobulins (Ig) sharing idiotypic determinants. The absence or presence of mutations in the variable heavy chain (VH) genes of the Ig locus is a well characterized prognostic factor in CLL, with the unmutated profile (i.e., <2% deviation from germ-line) associated with shorter overall survival [17].

In another study [24], we verified the existence of spontaneously occurring, hTERT-specific CTL cells in patients with telomerase-expressing leukemic cells. DC loaded with a 16aa peptide derived from either hTERT or with a Ras control peptide were used to stimulate autologous T-cells. Seven telomerase-positive patients, three telomerase-negative patients and three healthy control donors were tested. Proliferative T-cell response elicited by hTERT-loaded DC in telomerase-expressing patients was significantly higher than that elicited by the Ras peptide-loaded DC. The proliferative response was MHC class II but not MHC class I restricted. In six out of the seven telomerase-positive patients MHC class I-restricted, hTERT-specific cytotoxic T lymphocytes were also generated. In contrast, no significant hTERT-specific proliferative response relative to the ras peptide, could be detected in any of the telomerase-negative patients or in healthy control donors. No hTERT-specific cytotoxic T lymphocytes could be expanded in any of the three telomerase-negative patients.

These studies using hTERT as a model TAA indicated that antigen presentation by DC can reverse any putative immunological anergy and stimulate antileukemic responses.

Dendritic cells as antigen presenting platform of tumor-associated antigens

Despite having a normal expression of MHC class I and class II molecules on the cell surface, CLL cells are poor antigen-presenting cells as they lack co-stimulatory and cell-adhesion molecules such as CD80, CD86 and CD54 [4], which are essential to prime an effective T-cell response. Furthermore, CLL cells are known to secrete TGF-β, a factor known to have the potent immunosuppressive function [36].

Dendritic cells can serve the dual functions of cellular adjuvants as well as delivery systems for the immunogen. Immature DC endocytose antigens and process and present them in the context of major histocompatibility complex (MHC) molecules. Following maturation and activation, DC up-regulate adhesion and co-stimulatory molecules and acquire the capability of stimulating both the innate and adaptive immune system by interacting with CD4+ and CD8+ as well as NK cells. A number of preclinical studies and clinical trials (reviewed in [35]) have confirmed the utility of DC for active-specific immunotherapy of cancer. Typically the strategies involve loading DC with peptides derived from tumor-associated antigens (TAA), tumor lysates, RNA or DNA from tumors or fusing DC with tumor cells.

Clinical trials on active immunotherapy in CLL

There are few clinical trials investigating active immunotherapeutic approaches in CLL. Two of these trials involved the use of autologous CLL cells transfected with CD40-ligand (CD154) using an adenoviral vector. In the first study 11 patients were treated with autologous CLL

cells transduced with a replication-defective adenovirus construct to express murine CD154 [40]. Treated patients demonstrated increases in CD4 and CD8 T cells and had sustained reductions in lymphocyte counts as well as reductions in the size of the spleen and lymph nodes. In an unrelated study, 9 patients were administered autologous CLL cells transduced with adenovirus to express CD154 and interleukin 2. Most of the patients (7/9) demonstrated a transient increase in leukemia-reactive T cells and 3/9 patients had >50% reduction in the size of their lymph nodes [3].

Hus et al. [20, 21] have reported two clinical trials investigating DC pulsed with a tumor lysate as a vaccine in CLL. In the study published in 2005 [20], results with a total of nine patients were reported. The vaccine was based on allogeneic, HLA-mismatched DC generated from normal donors. Eight of the nine patients were reported in the analysis, of whom five had received allogeneic DC pulsed with autologous tumor lysates and three had received allo-DC pulsed with autologous apoptotic bodies. Some transient decreases in WBC or immune responses to the vaccines were noted. With the small number of patients, there was no clear indication whether apoptotic bodies or lysates were superior for stimulating antileukemic responses.

In a recent report [21], the same group of investigators published a study in which autologous DC pulsed with leukemia lysate were used as the cellular vaccine. Two separate methods were used to generate DC from monocyte precursors. The first two of twelve patients had DC generated from monocyte precursors selected by plastic adherence. For the next 10/12 patients, DC were generated ex vivo from monocytes enriched immunomagnetically from 150 ml of blood. The first two patients received five doses of vaccines whereas the next ten patients received eight doses of vaccines. Minor decreases in CD19+/CD5+ leukemic cells in five patients was noted as well as increase in circulating leukemia-specific CTL and a decrease in T_{regs}.

Identification of the optimal DC vaccine candidate

Our previous study [33] had indicated that several different clones of leukemia-reactive CD4 and CD8 cells were present in CLL patients leading us to speculate that a vaccination approach based on whole tumor cells was preferable to a single defined antigen. Additionally, this approach minimizes the potential selection of tumor antigen escape variants.

Validation of platform technologies for stimulating T cells with leukemia-associated antigens is central to DC-based immunotherapy of CLL. At the outset, DC were loaded with CLL antigens using a number of methods and compared for their ability to stimulate autologous T cells. In the first study, DC that had endocyted CLL apoptotic bodies (Apo-DC) were compared with DC-tumor cell hybrids [25]. The frequency of DC that had endocyted apoptotic bodies was higher than that of the DC-tumor cell hybrids (22.6 ± 6.2 vs. $10.5 \pm 2.6\%$). Additionally a proliferative response could be seen in 4/5 cases when using Apo-DC but not for fusion hybrids as stimulators of autologous T cells. Apo-DC and fusion hybrids induced an IFN-γ response in 4/5 and 3/5 cases, respectively, but IFN-γ secretion induced by Apo-DC was significantly higher than that by the cell hybrids. In a subsequent study, we evaluated Apo-DC with two other methods, i.e., tumor cell lysate or tumor cell RNA i [23]. Apo-DC were able to reproducibly induce a proliferative T-cell response, which was both MHC class I and II restricted. Additionally a significantly high number of IFN-γ-secreting T cells was detected when Apo-DC were used as stimulators as compared to tumor lysate or tumor RNA. The cytokine profile indicate a type I T-cell response. There was no difference in the rate of uptake of apoptotic bodies and tumor lysate (86 and 97%, respectively), but uptake of tumor RNA was low (19%). Based on these findings, the generation of Apo-DC was scaled up for clinical production of the cellular vaccine.

Large-scale production of the Apo-DC vaccine for clinical use: optimal enrichment of monocyte precursors

In the studies discussed above [23, 25, 32], CD14+ monocyte precursors were enriched with immunomagnetic beads from peripheral blood of CLL patients and used for ex vivo generation of DC.

CLL patients normally have high numbers of circulating leukemic cells in and relatively few monocytes. Enriching monocyte precursors from their peripheral blood in numbers sufficient for providing a clinical product is a technically challenging process. Since the DC production procedure is labor, time and cost intensive, it is preferable that an adequate number of DC required for repeated vaccinations be produced from a single leukapheresis product. By doing so, discomfort to the patient is reduced as is the batch-to-batch variation in the characteristics of the cellular vaccine.

A validated method for large-scale production of mature, monocyte-derived DC loaded with apoptotic CLL bodies has been previously published by our group. [22]. Since the vaccine is meant to be administered repeatedly to patients over a period of time, the aim of the study was also to investigate the impact of cryopreservation and recovery on the phenotype, viability and capacity of Apo-DC to induce T-cell responses.

Two different methods for enriching CD14+ monocyte precursors from CLL patients were compared: counterflow eluitration and CD14+ immunomagnetic separation. Eight CLL patients were leukapheresed and PBMC from two of them were separated in a counterflow centrifuge, while PBMC from the other six patients were immunomagnetically separated using the CliniMACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The monocyte fraction was used for generation of Apo-DC [22, 25].

Immunomagnetic separation yielded a higher number of CD14+ cells compared to counterflow eluitration. The monocyte purity was also higher with immunomagnetic separation as was the total number of viable DC generated.

For our clinical study, described subsequently, we established that a minimum of 50×10^6 Apo-DC would be needed. In our previous experience [1], the purity of the monocyte fraction after counterflow eluitration was shown to be mainly dependent on the percentage of monocytes in the starting leukapheresis material; a minimum of 4% CD14+ cells in the starting material was needed to achieve a satisfactory enrichment of monocyte precursors from cancer patients. We could show that a successful production of Apo-DC could be obtained with a minimum of 1% CD14+ monocyte precursors in the leukapheresis product using the CliniMACS system. The immunomagnetic enrichment method, however, was limited by the number of cells from the starting leukapheresis product that could be loaded on to the column. Nevertheless, adequate numbers of monocyte precursors could be enriched and Apo-DC could be generated from virtually every patient when two or three columns were used in parallel. The frequency of DC that endocyted apoptotic bodies, quantified by fluorescent membrane labeling was $77 \pm 4.3\%$. Upon thawing, $86 \pm 4.4\%$, of the cryopreserved Apo-DC were recovered with a viability of $90 \pm 2.8\%$. The phenotype of Apo-DC was not altered by cryopreservation and thawing, as shown by the expression of the markers HLA-DR, CD80, CD83 and CD86. Secretion of IL-12(p70) measured by ELISA was comparable in fresh and cryopreserved DC.

Apo-DC for the treatment of CLL patients: a phase I-II trial

Based on these as well as other preclinical studies, we designed a phase I/II clinical trial protocol which is currently ongoing. Previously untreated CLL patients with gradually increasing leukemic cell count (≈25% increase) during the last 6 months but no expected need of cytostatic treatment within at least the next 6 months, receive 1×10^7 Apo-DC for five immunizations. Patients who are in a long-term unmaintained response/plateau phase following previous chlorambucil therapy are also included. The vaccine dose was selected based on published data, in which doses of 1 x 10^6–5 x 10^7 DC have been used with no clear correlation of dose and adverse effects or immune responses. However, several studies have used 10^7 DC per immunization for induction of an immune response [8, 38, 41, 43].

The vaccine is administered every two weeks for 4 times, and a fifth administration is given 8 weeks after the fourth vaccination (week 14).

Primary objectives of the trial are: (1) determining the feasibility of generating dendritic cells loaded with apoptotic bodies (Apo-DC); (2) evaluating the safety of administering Apo-DC. Secondary objectives are to evaluate CLL-specific immune responses and clinical effectiveness.

The vaccine is administered intradermally (i.d.), in to the upper arm of the patient. Three cohorts of patients will be accrued stepwise, each composed of five patients. Eligible patients are accrued initially to cohort I alone. Accrual to cohorts II and III starts after the completion of cohort I if no dose limiting toxicities were noted (NCI grade III and IV toxicity).

The first cohort of patients receive Apo-DC alone. The second cohort receives Apo-DC+ GM-CSF (75 mg/day subcutaneously.), at the same site as Apo-DC, for four consecutive days starting the same day as Apo-DC administration. GM-CSF, together with autologous DC and irradiated tumors have been successfully described as a vaccination approach in advanced, treatment refractory melanoma [10]. GM-CSF is a critical cytokine for the differentiation and survival of dendritic cells and is known to promote the influx of mature DC to the site of injection as well as enhanced uptake of antigen [11]. Injection of GM-CSF has also been demonstrated to increase the number and activation state of DC in the draining lymph node of melanoma patients [42].

The third cohort will receive cyclophosphamide 300 mg/m^2 i.v. at day –2 at week 0, 6 and 14 and then the same treatment as cohort II with the aim to reduce T_{reg} cells, which may be present in CLL at high numbers.

Regulatory T cells are known to be increased in CLL patients compared to normal healthy individuals, particularly in patients with untreated or progressive disease [2]. This study also revealed that fludarabine-containing therapies greatly decreased the frequency of T_{regs}. The global T cell-suppressive property of fludarabine; however, makes it unsuitable for combination with vaccines. Cyclophosphamides was chosen in the present study based on previous reports that at 300 mg/mg^2, cyclophosphamide decreased the frequency and/or function of T_{regs} and increased the specific immune response to cancer vaccines [5, 19, 27].

Evaluation of clinical and immunological responses

The clinical efficacy of the vaccine will be evaluated as per International Workshop on Chronic Lymphocytic Leukemia (iwCLL) guidelines for CLL diagnosis and treatment

[16]. Analysis of the immune responses is performed by lymphocyte proliferation assay, IFN-γ ELISpot assay as well as cytokine secretion quantified by the Luminex® multiplex assay. Purified T cells are used as effectors and autologous CLL cells cultured for 48 h on a monolayer of huCD40L-transfected fibroblasts as targets. A positive immune response is defined as a $\geq$2-fold increase compared to pre-immunization values. A patient is defined as immune responder if the increase is noted in either the proliferation or ELISpot assay at $\geq$2 different time points during follow-up.

Conclusions

Despite technical difficulties in isolating monocyte precursors for DC generation, a large-scale production of individualized vaccines appears to be possible. At the present time no significant toxicity has been observed in any of the vaccinated patients and the trial is still in its early stages to comment definitively on any potential clinical effect. There is evidence that combining vaccination therapy with a T_{reg}-depleting agent (cyclophosphamide) may lead to improved clinical efficacy [9]. We intend to examine the clinical effects of Apo-DC vaccination together with T_{reg} depletion in the present clinical trial.

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