

Immunotherapy of acute myeloid leukaemia: development of a whole cell vaccine

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Acute myeloid leukaemia
4. Tumour associated antigens identified in acute myeloid leukaemia: providing targets for immunotherapy
5. T cell biology and tumour immunogene therapy
6. Transforming tumour cells into antigen presenting cells
7. Comparison of the efficacy of B7-1, B7-2 and 4-1BBL in a whole cell vaccine model
8. Combinations of costimulatory molecules and cytokines may have a synergistic effect
9. Technical issues regarding whole cell vaccines
10. Conclusions
11. Acknowledgements
12. References

1. ABSTRACT

Acute myeloid leukaemia (AML) is a difficult to treat disease and strategies, such as immunotherapy, which have the potential to eliminate residual tumour cells at first remission are required to reduce the incidence of relapse with its high associated mortality rates. T cells play an important role in tumor immunity and two signals are traditionally thought to be required to activate naïve T cells; signal one through the major histocompatibility :antigen : T-cell receptor complex and signal two through costimulation. Many tumor associated antigens have been identified in AML suggesting it may be possible to target the immune system of AML patients; however AML develops due to tumour and immune editing, two systems by which AML cells can escape immune surveillance. By genetically modifying AML cells to express costimulatory molecules and/or cytokines, it has been possible to transform AML cells into antigen presenting cells and this has the potential to re-activate the immune system in patients. Here we summarize the rationale for using a whole cell vaccine approach to treat AML, and discuss current progress in the field of whole cell vaccine development against AML.

2. INTRODUCTION

Cells of the immune system respond to “danger” signals, which can be provided by growing tumours as a consequence of the genotoxic stress of cellular transformation and the disruption of the surrounding microenvironment. Under ideal conditions, these signals will induce inflammation-activated innate-effector cells, which have antitumour activity, and stimulate professional antigen presenting cells (APC) to take up tumour associated antigens (TAAs). The APC will then migrate to the draining lymph nodes to trigger an adaptive immune response by B and T cells. Despite the fact that the immune system can detect some spontaneously arising tumour cells and eliminate them, genomic alterations appear to enable other tumour cells to express or delete a large amount of genes. These cells are then able to outgrow their normal counterparts and in the presence of natural selection escape immune surveillance, leading to the generation of transformed cells, which go on to form the tumour mass. It has been found that in many tumours, the major histocompatibility molecules (MHC), particular MHC class I, is down-regulated (1, 2), which leads to the diminished presentation of tumour antigens and is believed to be one of the mechanisms by which tumours escape the immune

Whole cell vaccine for AML

system. Loss of TAA expression is another immune escape mechanism observed in tumour cells. Yee *et al.* (3) demonstrated the specific loss of cognate melanoma antigen expression in tumours from relapsed patients following the adoptive transfer of melanoma antigen specific CD8⁺ T cells. Tumours may secrete or express molecules which inhibit immune responses such as TGFbeta which has been shown to be produced by various tumours (4-6). Tumours may also induce T cell tolerance, and as T cells play a very central role in tumour immunity, induction of tolerance prevents an active T cell response against the tumour (7-10). The aim of tumour immunotherapy is to generate a long lasting functional immunity in the host which can kill tumour cells and strategies must be developed to overcome the mechanisms by which tumours escape the immune system. The whole cell vaccine is one approach that has been designed for this purpose.

3. ACUTE MYELOID LEUKEMIA

AML is an aggressive cancer in adults. AML accounts for approximately 30% of all adult leukaemias, with 18,300 new cases being diagnosed in Europe each year. AML accounts for approximately 0.6% of all cancers. Despite new treatment protocols being developed during the last 20 years, the disease is still associated with high morbidity rates. In Europe, survival rates for adults with AML at 1 year were 34% and 15% at 5 years during the period of 1990-1994. The five-year survival rate of AML is found to decrease with age from 37% to 2%; from the youngest age group (15-45 years old) to the oldest age group of patients (75 years old or over) respectively (11). AML is a heterogeneous disease in terms of morphological, biological and molecular characteristics. Attempts have been made to classify the disease. The historically accepted subgrouping of AML was the French-American-British classification system (12), which grouped patient samples based on morphological and cytochemical criteria. However this was superseded by the World Health Organization classification system which takes into account additional, clinically relevant, factors such as genetics and immunophenotype (13). Untreated AML can be rapidly fatal over a short period of days to weeks depending on the blast count in the peripheral blood and the ensuing presence of complications such as marrow failure, tissue infiltration and/or hyperuricaemia. Chemotherapy is one of the most common treatments for AML. However, for patients under 50 years old, the most effective therapy to date is allogeneic stem cell transplantation (SCT), which allows an intensification of treatment and has a subsequent relapse risk of only 20%-25%. In addition the “graft-versus-leukaemia” effect reduces the relapse risk. However, patients do not always have an eligible donor with matching or partially-matching MHC molecules. For those patients who have a suitable donor, problems such as regimen-related toxicity, graft-versus-host disease and infection, result in 20-25% mortality after allogeneic SCT (11). The outcome of allogeneic SCTs are related to the age of patient: 5 year survival rates for patients under 20 years old are 60% and reduce to 40% for patients over 20 years old (11). Other treatment options need to be considered for

patients not eligible for allogeneic SCT. Immunotherapy is one of those options.

4. TUMOUR ASSOCIATED ANTIGENS IDENTIFIED IN ACUTE MYELOID LEUKAEMIA: PROVIDING TARGETS FOR IMMUNOTHERAPY

Cancer is a disease caused by changes in the expressed genes (14). Genes whose levels of expression are altered, often by being elevated, provide a source of TAAs. Like many other cancers, a number of TAAs have been identified in AML. Cancer-testis antigens provide a very appealing source of antigens for immunotherapy because they are expressed in tumour cells but not in normal tissues, except immunological protected sites such as the testis and placenta. Immunologically protected sites do not express MHC class I and therefore are not targeted by the “self-antigen” MHC class I mediated cellular immune response. The abnormal expression of cancer-testis antigens in tumour cells appears to be, at least in part, due to demethylation of the promoters of the genes concerned (15, 16). Using microarray on 124 AML patient samples, genes encoding a range of cancer-testis antigens (*RAGE1*, *MGEA6*, *SYCP1*, *SAGE*, *GAGE-D2*, *GAGE3*, *GAGE5*, *MAGE-C1*, and *CTP11*) were found to be expressed in AML patients but not normal donor samples (17). In another microarray study 116 AML patient samples were analysed and high expression of at least one of the three following TAAs, *RHAMM/HMMR*, *PRAME* or *G250/CA9*, were found to provide a strong favorable prognostic indicator. Greiner *et al.*, also showed that most of the AML patient samples analysed had specific T cell responses against these three antigens (18). RT-PCR screening and the serological analysis of recombinant cDNA expression libraries (SEREX) have also been used to identify new targets for AML immunotherapy such as *HAGE* (19), *PASD1* and *SSX2IP* (20), respectively. These findings and others suggest that AML cells do express TAAs, and it is hoped these TAAs can be targeted for immunotherapy.

5. T CELL BIOLOGY AND TUMOUR IMMUNOGENE THERAPY

T cells remain very attractive instigators of anti-tumour immunity due to their ability to remember a stimulus and respond more vigorously to repeated exposure by virtue of their memory response. In order to activate naïve T cells, two key signals are required. Signal one is provided through the TCR while signal two is that of costimulation. To generate anti-tumour cellular immunity, naïve T cells must encounter tumour antigens. Memory T cells rather than naïve T cells patrol the periphery, while naïve T cells travel to the peripheral lymph nodes. Due to this T cell recirculation pattern, it is not possible for the naïve T cells to encounter tumour cells outside of the lymphoid tissue (21). Eventually, tumour-specific T cell responses may result from the cross priming of T cells with tumour cell-derived antigens presented on dendritic cells (DCs) (22, 23). DCs take-up antigens and degrade them into peptides, which are loaded onto MHC molecules and are presented to antigen specific T cells (24). Effective naïve T cell stimulation only occurs in parallel to antigen

Whole cell vaccine for AML

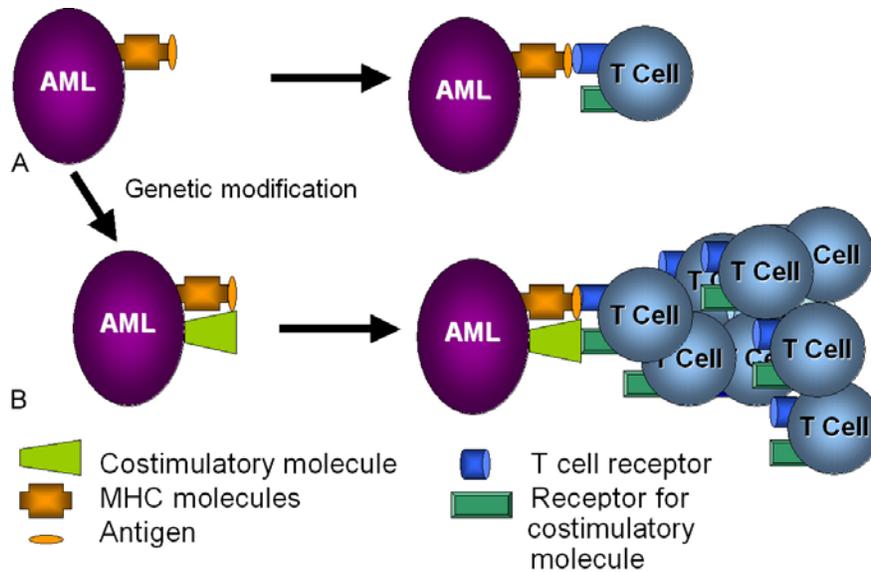


Figure 1. Diagrammatic representation of the way in which modified AML cells can act as APCs during the activation naïve T cells. (a) AML cells express TAA, however they tend not to express costimulatory molecules, thus naïve T cells can not be activated; (b) In a whole cell vaccine modified AML cells are engineered to express a costimulatory molecule, thus the T cells receive both signal one (MHC: Antigen: T cell receptor) and signal two (costimulatory signal), and can be activated.

uptake, when DCs undergo maturation, which is triggered by immune stimuli through toll like receptors or by inflammatory cytokines such as TNF- α , IL-1 β or IL-6 (25). Mature DCs migrate to the draining lymph nodes and secrete cytokines such as IFN- γ and IL-12 (26). Maturation also increases the levels of surface expression of various costimulatory molecules such as B7-1, B7-2 and CD40. This cross priming mechanism for stimulating naïve T cells is believed to play a role in the induction of tumour-specific T cell responses.

6. TRANSFORMING TUMOUR CELLS INTO ANTIGEN PRESENTING CELLS

Costimulatory molecules, in the form of cDNA, have been used to modify tumour cells and generate whole cell vaccines. The rationale behind this treatment is that if a tumour cell already expresses a 'signature' of mostly undefined tumour antigens, then the universal modification of the tumour cell, with a costimulatory molecule, will improve its ability to act as an APC, while avoiding the need to define these the tumour antigen (s). This strategy also avoids the limitations associated with having to identify MHC restricted epitopes and in some cases their limited applicability (27). In addition costimulatory molecule transfection provides an abundance of signal two (Figure 1).

Various methods have been used to transduce human cells so that the transgene of interest is inserted and expressed in the cells. These methods include electroporation, lipofection, retroviral and adenoviral transduction. However the transduction of primary human AML cells has always been difficult. Electroporation has a very low transfection efficiency, in which a lot of cells are

killed during the electroporation step. Retroviral vectors can only infect dividing cells (28), so for primary human AML cells which tend not to divide *ex vivo* even in the presence of cytokines (29), the success of this method has been limited. Recently, a lentivirus based method has been developed to transduce human AML cell lines with costimulatory molecules (30). To minimize the potential risk of generating a replication-competent lentivirus through the use of HIV-derived sequences, third generation self-inactivating vectors were used. In this system, a three-plasmid expression system, pCMV- Δ D8.9, pMD.G and HR'SINctwSV was used to generate infectious particles through the transient transfection of the human 293T cell line. The advantage of using lentiviral vectors is that these viruses can transduce resting cells, due to the presence of the HIV matrix protein and the accessory protein Vpr (31, 32). The incorporation of the HIV central polypurine tract and termination sequences improves nuclear import in non-permissive targets (33, 34). Furthermore the presence of the woodchuck hepatitis virus posttranscriptional regulatory element improves intracellular messenger RNA stability (35). The effectiveness of lentiviral transduction on primary leukemic cells has been demonstrated elsewhere (29, 30).

7. COMPARISON OF THE EFFICACY OF B7-1, B7-2 AND 4-1BBL IN A WHOLE CELL VACCINE MODEL

Several investigators have investigated the capacity of the co-stimulatory molecules B7-1 and/or B7-2 to convert leukaemia cells into whole cell vaccines in animal models. B7-1 and B7-2 have been shown to interact with the CD28 signaling pathway and their costimulatory capacity is limited through subsequent CTLA4 interactions (reviewed in detail in 36, 37). However, B7-1 and B7-2

Whole cell vaccine for AML

were found to vary in efficacy depending on the tumor cell line model. For example Dunussi-Joannopoulos *et al.* performed intensive studies on radiation induced murine AML cells (38, 39). In their AML model, they found that AML cells transduced with either B7-1 or B7-2 had reduced tumorigenicity compared with parental cells, although only B7-1 elicited systemic immunity. In studies using 32Dp210 cells, B7-1 or B7-2 transfection led to the rejection of the modified tumour cells by the syngeneic host, although 32Dp210/B7-1 was found to be superior to B7-2 in its capacity to protect against wild type tumour challenge and eradicate minimal residual disease (40). Other studies have shown that B7-2 was better than B7-1 at converting myeloid leukaemia cells into a whole cell vaccine, which is consistent with our finding in the 32Dc-kit model (Cheuk *et al.*, in preparation). Labelle *et al.* showed that elevating B7-1 and B7-2 expression in a spontaneously arising murine myelogenous leukaemia line, C1498, induced syngeneic CD8⁺ T cells to kill parental C1498 cells *in vitro*. When injected s.c. into a syngeneic host, B7-1 expressing C1498 grew progressively whereas B7-2 expressing C1498 (C1498/B7-2) tumours completely regressed. Spontaneous rejection of C1498/B7-2 cells resulted in immunity against subsequent challenge with the parental cells (41). It should be noted that the efficacy of either B7-1 or B7-2 to convert tumour cells into a whole cell vaccine appeared to depend on the immunogenicity of the cell line model used (42, 43).

We transfected human AML cells with B7-1, B7-2 or 4-1BBL, and human allogeneic MLRs. In our unpublished data (Cheuk *et al.*, in preparation) we found that it was not easy to compare the efficacy of different costimulatory molecules in primary human AML samples, as each tumour has its own molecular signature and the T cell proliferative response was unpredictable between samples although reproducible within samples. 4-1BB ligand (4-1BBL) is a member of the tumour necrosis factor receptor family and is a costimulatory molecule which serves to amplify and diversify the T cell immune response (reviewed in 44, 45). To date only a small number of studies have directly compared the costimulatory molecule 4-1BBL with B7-1 and/or B7-2 within a single tumour model system. Guinn *et al.*, showed that in the murine A20, B-cell lymphoma model, which had innate B7-1 and B7-2 expression, transfection with 4-1BBL could create a whole cell tumour vaccine, which was more efficacious at inducing anti-tumour immunity than transfection of the same cells with either B7-1 or B7-2 alone (46). This group showed that in the A20 model, protective immunity against parental tumors involved both the 4-1BBL and CD28 pathways (46). Coexpression of 4-1BBL and B7-1 in the poorly immunogenic AG104A sarcoma enhanced the induction of effector CD8⁺ T cells which could reject even the wild-type AG104A tumour while neither 4-1BBL nor B7-1 single transfectants were as effective (47). NRS1 is a murine squamous cell carcinoma that constitutively expresses B7-1 at high levels, yet it was the introduction of the 4-1BBL cDNA that efficiently elicited anti-tumour immune responses in syngeneic mice which then acquired specific immunity against the wild-type tumour (47). In general, 4-1BB receptors are only induced on activated T

cells and the costimulatory pathway, CD28:B7 appears to be necessary to activate T cells through primary responses or for a secondary response in 4-1BB knockout mice (48, 49). 4-1BB:4-1BBL acts as an amplifier of existing costimulatory signals (46) but CD28:B7 is still the primary and generally the most effective mode of initiating an immune response (at least in anti-tumour responses). All of these data suggest a synergistic effect between the CD28:B7 and 4-1BB:4-1BBL costimulatory pathways. The CD28:B7 pathway is not required to protect the host against modified tumour, but the CD28:B7 pathway is required for memory responses (50).

8. COMBINATIONS OF COSTIMULATORY MOLECULES AND CYTOKINES MAY HAVE A SYNERGISTIC EFFECT

One way to increase the efficacy of a whole cell vaccine is to use immunoregulatory cytokines in cooperation with costimulatory molecules, which can synergistically enhance the efficacy of a whole cell vaccine. IL-2 has been shown to be a main cytokine that induces T cells in cell cycle from G₁ to S, which is followed by T cell proliferation (51). GM-CSF has been described as a factor supporting clonal growth and differentiation of bone marrow progenitor cells, granulocytes and monocytes. It also stimulates the anti-tumour activities of neutrophils, eosinophils and macrophages, and stimulates the maturation of DCs, up-regulates the surface expression of costimulatory molecules, and enhances the DC antigen presentation efficiency (52). IL-12 has been used as an adjuvant in whole cell vaccines. IL-12 is a cytokine with a broad spectrum of activities and is produced by activated macrophages, B cells and DCs. Among other activities, IL-12 stimulates the proliferation and cytotoxic activity of NK and T cells (52). In cooperation with costimulatory molecules, cytokines can further amplify the immune response and provides a powerful tool for enhancing tumour immunotherapy. Chan *et al* have combined B7-1 with IL-2 in a fusogene lentiviral vector which leads to expression of biologically active membrane-bound B7.1 and secreted IL-2. They then transduced primary AML cells and showed that the combination of B7-1 and IL-2 were more effective than either molecule alone at stimulating T cell proliferation and in some experiments the combination was synergistic at inducing T cell responses (30).

9. TECHNICAL ISSUES REGARDING WHOLE CELL VACCINES

There are technical difficulties associated with whole cell vaccine therapies. AML primary tumour cells are very difficult to culture *ex vivo* and it has been onerous to obtain a large enough gene modified population for transplantation back into patients (53). Most whole cell vaccine models in mice have involved injecting the modified live tumour cells into mice in the first instance and then challenging with primary tumour cells. In most animal models, a "live" whole cell vaccine is used rather

Whole cell vaccine for AML

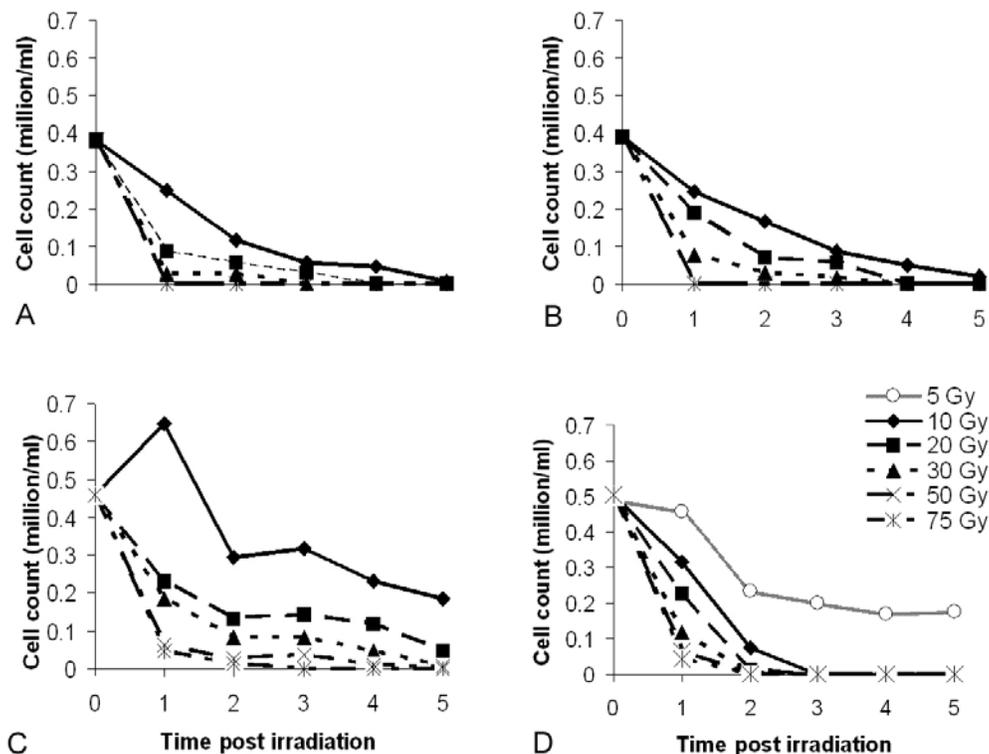


Figure 2. AML cell lines growth patterns after receiving each of a range of doses of irradiation. Aliquots of 5 ml of 0.5×10^6 cells/ml of (a) HL60; (b) NB4; (c) U937 or (d) P39, were irradiated at different doses represented by different style of lines (as shown in key). The cells were then transferred and cultured in 6 well plates. Cell counts were performed in duplicate at 24 hour intervals. The X-axis shows time in days since the start of the culture in 6 well plates while the Y-axis shows cell count/ml. The median cell count and standard deviation for each cell line is shown.

than an “inactivated” whole cell vaccine. The reason behind this, is that a live vaccine is easy to prepare, and the immune response can be easily monitored by directly measuring modified tumour growth. However, this “live” whole cell vaccine can potentially revert back to the parental tumour cell genotype as shown previously (46). Guinn *et al* found that tumours which formed in mice, injected with the A20 cell modified to express B7-2, had no B7-2 expression when excised and analysed without being passaged. In our unpublished data (Cheuk *et al*, in preparation) we also found that bone marrow cells from 32Dc-kit injected mice could be killed by Zeocin, the selectable marker for the plasmid cassette carrying the costimulatory molecule cDNA but not with Neomycin, the selectable marker on the c-kit retrovirus. In addition, a live vaccine does not recapitulate the therapy setting in which patients will be treated in clinical trials with a whole cell vaccine. A “live” whole cell vaccine cannot be used in humans, and only an inactivated “dying” whole cell vaccine can be used. Irradiation is a commonly accepted method for inactivating tumour cells in whole cell vaccines, however the irradiation dosage will have to be chosen carefully. High doses of irradiation will kill the cells which constitute the vaccine straight away, such that when these cells are injected into its host, no immune response against the tumour will be initiated. However, if the irradiation dosage is too low, the cells have the potential to fail to die.

Moreover, cancer is a heterogeneous disease, which means that the dosage of irradiation necessary to kill human tumour cells may vary from cell line to cell line (Figure 2) and patient to patient even within the same disease.

Cells of the immune system are constantly renewed from haematopoietic stem cells. With age, a reduction in the overall capacity for renewal of these cells has been observed (54, 55). Patients with AML tend to be of an advanced age, when there is a decline in naïve T cell numbers observed due to diminished thymopoiesis (54). At this advanced age, T cell responses against cancer vaccines would be expected to depend on recognition of the tumour cells by memory cells (56). T cells in response to the vaccine may not be the “best-fit” that may have been selected from naïve T cells in earlier life (56). The design of any immunotherapy protocol for AML will need to overcome age related issues. In a study by Bansal-Pakala & Croft (57), they showed that the engagement of 4-1BB can amplify T cell responses in aged mice, and this may help to solve problems resulting from trying to stimulate an aging immune system to kill tumour cells.

10. CONCLUSIONS

AML is a heterogeneous disease, which develops its own “signature” of antigen expression in different

Whole cell vaccine for AML

patients within the same subgroup of diseases. In order to provide a more complete coverage of the antigens expressed and still be able to stimulate the immune system in a wider range of patients, the modification of tumour cells with combinations of different costimulatory molecules and cytokines may provide an effective whole cell vaccine without the need to define the tumour antigens expressed. With advances in technology and the experience learned from animal studies, it is likely that whole cell vaccine approach will soon be used to treat AML patients (58).

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Whole cell vaccine for AML

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Abbreviations: 4-1BBL: 4-1BB ligand; AML: acute myeloid leukaemia; APC: antigen presenting cells; DC: dendritic cell; MHC: major histocompatibility complex; SCT: stem cell transplant; TAA: tumor associated antigen

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