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An immune edited tumour versus a tumour edited immune system: prospects for immune therapy of acute myeloid leukaemia

Abstract Cell based therapies for acute myeloid leukaemia (AML) have made significant progress in the last decade benefiting the prognosis and survival of patients with this aggressive form of leukaemia. Due to advances in haematopoietic stem cell transplantation (HSCT) and particularly the advent of reduced intensity conditioning (RIC), the scope of transplantation has now extended to those patients previously ineligible due to age and health restrictions and has been associated with a decrease in transplant related mortality. The apparent graft versus leukaemia (GvL) effect observed following HSCT demonstrates the potential of the immune system to target and eradicate AML cells. Building on previously published pre-clinical studies by ourselves and others, we are now initiating a Phase I clinical study in which lentiviral vectors are used to genetically modify AML cells to express B7.1 (CD80) and IL-2. By combining allogeneic HSCT with immunisation, using the autologous AML cells expressing B7.1 and IL-2, we hope to stimulate immune eradication of residual AML cells in poor prognosis patients that have achieved donor chimerism. In this report we describe the background to cell therapy based approaches for AML, and discuss difficulties associated with the deployment of a chronically stimulated, hence exhausted/depleted immune system to eradicate tumour cells that have already escaped immune surveillance.

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Abbreviations HSCT: Haematopoietic stem cell transplant · AML: Acute myeloid leukaemia · GvL: Graft versus leukaemia · DLI: Donor leukocyte infusion · VUD: Volunteer unrelated donor · GvHD: Graft versus host disease · MDS: Myelodysplastic syndrome · RIC: Reduced intensity conditioning · APC: Antigen presenting cell · mHA: Minor histocompatibility antigens · LD-DC: Leukaemia derived-dendritic cell · CTL: Cytotoxic T lymphocytes

Introduction

Advances in the field of tumour immunobiology have improved our understanding of the role of the immune system in preventing tumour development. We now understand that tumour antigens can be recognised by the immune system and that natural immune responses do occur against tumour cells. These responses, involving both the innate and adaptive arms of the immune system, can lead to the elimination of tumour cells. This occurrence is best exemplified by the increased rate of tumour development in immuno-compromised individuals [1]. Where the immune system can mount a response against tumour cells, then the development of antigen-specific CTL activities and the generation of memory T cells and protective immunity can be detected. However, tumour cells are able to escape immune surveillance. Immunological responses against tumour cells are likely to be most effective against the most immunogenic of the tumour cell population, and least effective against the escape mutants that have evolved various means of camouflage or resistance to immune mediated toxicity. This is a continuously evolving process and the most vulnerable of the tumour cells are likely to be the most efficiently eradicated, leaving an evolving population of tumour cells with increasingly more effective means of combating immune mediated eradication. This phenomenon, named "immune-editing" of tumour cells by Robert Schreiber, has important implications for the development of immune therapy strategies that may

have any realistic chance of therapeutic benefit (see Schreiber et al. [2, 3] for recent reviews).

Tumour/immune interactions also affects the immune system. The continued survival of the tumour and chronic immune stimulation could result in the eventual exhaustion and even clonal deletion of the most stimulated components of the immune system (e.g. the corresponding CTLs). In addition, T cell encounters with tumour antigens in the absence of adequate co-stimulatory and helper functions could result in anergy and tolerance to these antigens [4, 5]. These tumour mediated influences on the immune system, the "tumour editing" of the immune system, has important implications for the development of tumour immune therapy. For instance, the strongest tumour antigens may not provide the best vaccination targets, as the corresponding T cells may be very effectively exhausted and/or clonally deleted. In contrast, some of the weaker tumour antigens, which may have failed to spontaneously induce an effective immunological response, may be able to draw on a reserve of unstimulated cells which may then be effectively stimulated in response to vaccination in the presence of the appropriate adjuvants and helper functions.

Acute myeloid leukaemia as a target for immune therapy

Acute myeloid leukaemia (AML) is one of the most aggressive forms of haematological malignancies affecting both the young and the old. This disease has a particularly poor prognosis (11% survival at 5 years post-diagnosis) in patients over 55 years of age, due to these patients' difficulties in tolerating intensive chemotherapy or pre-transplant conditioning, and the likely absence of suitable transplant donors. Despite recent advances in chemotherapy and HSCT, particularly the advent of RIC, the survival rates amongst AML patients remain poor. It is for these patients that immunisation with AML cells expressing immune stimulatory factors may offer a new adjuvant therapy option.

A wealth of evidence exists in the literature to show that patients can generate immune responses against antigens expressed by their own tumour cells. AML is no exception and the presence of pre-treatment autoantigens and spontaneous T cell responses against tumour antigens have been demonstrated. Examples of some of the autoantigens identified in AML include RHAMM [6], MPP11 [7], SSX2IP and the cancer-testis antigen PASD1 [8]. WT1 is detectable in many forms of cancer (lung, breast, ovarian) and has been shown to be expressed at high levels in haematopoietic malignancies and to play a role in the pathogenesis of several leukaemias including the development of AML [9]. A number of studies have demonstrated the presence of antibodies against WT1 in AML patients [10–12]. The first evidence of spontaneous T cell responses against defined antigens in AML patients came from a study by Scheibenbogen et al. [13] when they described the presence of granzyme B positive $CD8^+$ T cell responses against proteinase 3 and WT1. More recently the presence of RHAMM peptide-specific effector T cells in the peripheral blood of patients with AML was shown using HLA-A2/R3 tetramers in a population of $CCR7-CD45RA^+$ cells. Spontaneously occurring T cell immune responses against BCL-2 have also been documented in patients with AML [14]. A number of other AML associated antigens ranging from mutated oncogenes to products of chromosomal translocations have been identified that can potentially act as tumour antigens.

The identification of AML associated antigens provides opportunities to generate tumour specific cytotoxic T cells. However this potential remains unrealised, not least because of technological difficulties in the expansion of functionally cytotoxic T cells. In addition, a fundamental hurdle in cancer immune therapy remains in the rejection of an immune edited tumour (thus selected for loss of susceptibility to immune rejection) by a tumour edited immune system, already depleted of its most potent effector cells by chronic stimulation mediated exhaustion and clonal deletion.

Immunotherapy for AML

One possible approach to "re-activating" the immunogenicity of tumour cells is to turn them into effective antigen presenting cells (APCs). AML blasts are of the same developmental lineage as APC and therefore express a range of molecules required for the direct presentation of antigens to T-cells. Important amongst these are the expression of both MHC class I and class II for the activation of $CD8^+$ and $CD4^+$ cells, appropriate adhesion molecules and some co-stimulatory signals. However, AML blasts invariably lack the expression of a key co-stimulatory factor—B7.1 (also known as CD80) [15]. Several promising approaches have been developed for the immunotherapy of AML, including the modification of autologous AML blasts to act as a whole cell vaccine. This type of cellular therapy has included the in vitro differentiation of AML cells to a "leukaemia derived-dendritic cell" phenotype (LD-DC). Although LD-DCs have been demonstrated to stimulate autologous T-cell activation [16–19], efficient LD-DC generation has been variable in different studies, and LD-DCs may not be able to stimulate CTL activity in autologous T-cells [20, 21]. Alternatively, myeloid leukaemia cells have been genetically modified in order to counteract their inherent immune suppressive activity and to achieve T-cell stimulation [22–24]. Several studies in solid tumour models have now shown that the combined expression of B7.1 and stimulatory cytokines such as IL-2, IL-7 and GM-CSF, make tumour cells potent stimulators of both allogeneic and autologous T-cells. Where examined, the responding T-cells appear to have MHC restricted and antigen specific CTL activity [25–31].

In a recent clinical study, AML patients with chemotherapy induced cytopenia show reduced in vitro T cell responsiveness compared to normal subjects. However, this can be restored to normal levels by B7.1 co-stimulation [32], making it a highly promising molecule for immunogene therapy of AML.

T cell stimulation by B7.1/IL-2 transduced AML cells

The absence of co-stimulation and the secretion of immunosuppressive factors are ways in which AML cells evade immune attack [33, 34]. However, it may be possible to overcome both effects by genetically modifying AML cells to express a potent co-stimulatory molecule such as B7.1, and to also provide additional helper functions by the expression of a pro-inflammatory cytokine such as IL-2. As a single agent IL-2 has been shown to reverse anergy both in vitro and in vivo [35] (Fig. 1). We and others have previously shown that B7.1/IL-2 expressing tumour cells can stimulate T-cell [23, 36–41] and NK cell [42, 43] responses against unmodified tumour cells, resulting in the immune mediated rejection of tumours and a prolonged survival in vaccinated animals ([44–46], see [47] for review). In human in vitro studies we have investigated the immunotherapeutic potential of primary AML blasts transduced with B7.1 and IL-2 [22, 48, 49]. When compared with
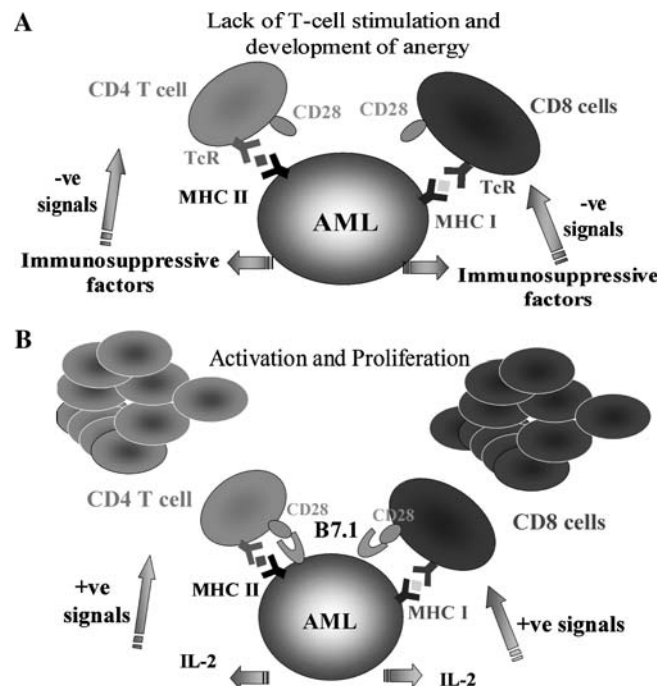

Fig. 1 Strategies for AML-immune activation. **a** Immune evasion by AML. In the absence of co-stimulation, T cells develop anergy towards AML. This is augmented by the secretion of immunosuppressive factors, which further inhibits immune responses against AML. **b** Genetically modified AML cells for immune activation. B7.1 (CD80) and IL-2 gene transduction provides the essential co-stimulatory signal and IL-2 for induction of anti-leukaemia immunological responses

single B7.1 or IL-2 gene modification or unmodified controls, B7.1/IL-2 transduction provided the best T-cell stimulation in allogeneic (threefold increase over unmodified control), autologous (7.4-fold) and chimeric settings (34.7-fold). The T-cell stimulation could be partially blocked by anti-B7.1 and anti-MHC class I antibodies [49]. This T-cell stimulation coincided with the expansion of activated CD8$^+$ cells and increased secretion of Th1 cytokines (IFN-γ, TNF-α and IL-2). Importantly, this was observed with T cells from post-transplanted patients who had achieved donor chimerism. While this is encouraging, unless CTL activity against primary human AML cells can be demonstrated, hopes for successful immunotherapy of this disease must remain cautious. There are few reports of immunotherapy strategies successfully initiating CTL activity against AML cells in vitro [18, 50]. The absence of CTL activity could be due to the inherent resistance of primary human AML cells to cell mediated lysis. We have previously shown that AML cells secrete an inhibitory factor that is capable of limiting the secretion of Th1 cytokines by T cells and lowering T cell proliferative responses [33, 34]. Alternatively it could be that PBLs obtained from AML patients, although capable of some degree of proliferation and cytokine release, may lack effective end stage lytic function. However, our current studies using B7.1/IL-2 modified AML cells suggest that, at least in vitro, autologous cytolytic T cells can be generated against AML cells (Hardwick et al., manuscript in preparation). Therefore, the combined expression of B7.1/IL-2 by genetically modified AML cells could enable the stimulation of an effective lytic response.

An efficient gene transfer protocol for AML blasts to co-express B7.1 and IL-2

Until recently, expression of transgenes in human AML blasts have been limited to the use of retroviral [22, 51] and adenoviral vectors [51]. These transductions had low efficiencies due to the failure of primary AML blasts to cycle in culture, limiting transduction efficiencies and variable coxackie adenovirus receptor expression [52, 53], respectively. In addition, the use of vectors based on herpes simplex virus and adeno-associated virus give highly variable transduction efficiencies (our own unpublished observations), all of which has seriously hampered the development of immune gene therapy for AML. Ex vivo gene transfer presents many practical advantages over in vivo gene delivery in that isolating and targeting of the specific tissue type or organ is relatively easy. In contrast to classical retroviral vectors, lentiviral vectors readily transduce mitotic as well as a number of post-mitotic targets, owing to the HIV matrix protein and the accessory protein Vpr [54, 55]. Although lentiviral vectors have mainly been used for the long-term transduction of non-dividing cells, it has proved to be highly effective, in comparison to other commonly used vectors, in the transduction of primary AML blasts

[23, 24, 56–58]. This is mainly due to the ability to pseudotype lentiviral vectors with VSV-G envelope protein for ubiquitous cell entry, the stability of VSV-G also allows centrifugal vector concentration and the capacity of lentiviral vectors to infect non-dividing cells is advantageous for the transduction of primary cells ex vivo. It is possible to remove viral genes from the vector and to provide the required products in trans in the vector producing cells, allowing the assessment of the therapeutic potential of the transgenes concerned (e.g. B7.1 and IL-2) without the risk of subverting immunological responses against viral proteins at the expense of the tumour-associated antigens. To this end, we have constructed second- and third-generation self-inactivating lentiviral vectors incorporating cppt/cts, a wpre enhancer element and a myeloid enhanced promoter—the U3 region of spleen focus forming virus [49, 59]. Transduction of primary AML blasts of multiple FAB subtypes can be achieved with an efficiency of 40–100% following a single round of infection at an MOI of 10.

To produce viral vectors at high titres, we have developed a highly efficient virus concentration strategy by capturing retroviral particles using conjugated paramagnetic microparticles (PMP) [60]. This has now been further developed for lentiviral vectors pseudotyped with both the VSV-G and amphotropic envelope [49, 61]. The result is a 380-fold increase in titre for VSV-G and 3,600-fold for amphotropic envelope, providing titres in the order of 10^9 cfu/ml (Fig. 2b exemplifies a typical PMP-mediated concentration). In addition, these PMP-concentrated lentiviral vectors displayed an increased infectivity of primary AML blasts. Compared with conventional ultracentrifugation based concentration strategies, we find a higher infection rate as a function of viral capsid protein p24 concentration, demonstrating an improvement in transduction efficiency.

To facilitate the efficient co-transduction of both B7.1 and IL-2, we have developed a "fusagene" strategy by which multiple gene products are expressed as a fusion protein from a single cistron [48]. The fusion protein contains recognition sequences for the golgi located, ubiquitous endoprotease and furin. Furin cleavage results in the generation of biologically active constituents from the fusion protein. Thus soluble IL-2 is released extracellularly whilst transmembrane B7.1 remains anchored at the cell surface. This strategy avoids the problems associated with transduction of AML blasts with two single gene vectors, or promoter interference when using two separate promoters. A well established alternative strategy is the use of internal ribosome entry sites (IRES). However, the instability of vectors with more than one IRES restricts the suitability of IRES vectors to express multiple genes.

Allogeneic HSCT and RIC

A lethargic immune system, exhausted by "tumour editing" of the immunological response and faced with
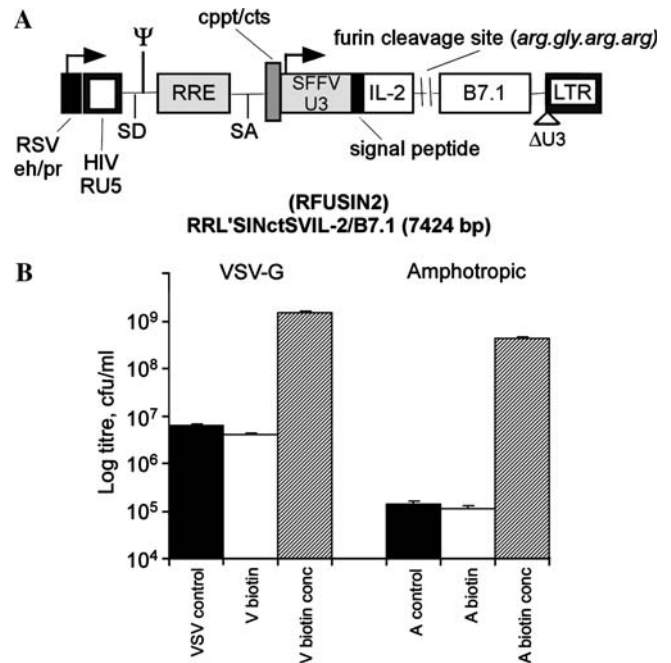

Fig. 2 Third generation self-inactivating lentiviral vector. **a** Backbone encoding B7.1 (CD80) and IL-2 cDNAs in a monocistronic expression cassette driven by a myeloid efficient promoter derived from spleen focus forming virus (*SFFV*). The IL-2/B7.1 fusion protein is separated by a recognition sequence for the endoprotease–furin, which cleaves to generate the biologically active constituents of soluble IL-2 and transmembrane B7.1. **b** Magnetic concentration following conjugation with paramagnetic microparticles (*PMP*). Titres presented as cfu/ml following infection and selection of infected leukaemic K562 cells in a soft agar cloning assay. *VSV or A control* unconcentrated, *V or A biotin* unconjugated control titre, *V or A biotin conc* PMP mediated concentrated samples (methodology described by Chan et al. [49])

the outgrowth of "immune edited" tumour cells, is unlikely to be able to mount a therapeutically effective immune response. However, haematopoietic stem cell transplantation (HSCT) mediates installation of an "unedited" immune system, and reduction of the mass of the tumour target (e.g. by chemotherapy mediated induction of albeit a transitory remission), may provide an opportunity for the donor derived immune system to eradicate the residual tumour.

The single most effective therapy for intermediate and poor risk AML is allogeneic HSCT. The therapeutic GvL effect that follows HSCT and donor leukocyte infusions (DLI) demonstrates the pivotal role that the immune system can play in eradicating leukaemia cells. This is best demonstrated by the reduced risk of relapse in patients receiving allogeneic HSCT, compared to autologous HSCT, showing both the ability of the immune system to recognise AML cells, and the susceptibility of AML cells to be eradicated by allogeneic T-cells. The involvement of T-cells in this response is further shown by the increased risk of relapse by T-cell depletion of the transplanted HSCT and by the efficacy of DLI.

Reduced intensity conditioning prior to allogeneic HSCT has considerably reduced transplant related mortality, thus extending the eligibility criteria to AML patients that were previously at too great a risk of transplant related toxicity. The additional inclusion of anti-CD52 (CAMPATH-1) in the transplant conditioning regimen has allowed efficient in vivo purging of donor T cells, with the consequent reduction in graft versus host disease (GvHD). We have recently reported the results of allografting following conditioning with fludarabine, busulphan and CAMPATH-1H in 62 patients with high risk myelodysplastic syndrome (MDS) and AML ([24] with matched sibling donors and 32 volunteer unrelated donors, VUD) [62]. The median age for sibling recipients was 56 (41–70) and for VUD recipients 52 (22–65) with a median follow up of 524 days (93–1,392) and 420 days (53–1,495), respectively. The non-relapse mortality at day 100, 200 and 360 days were 0, 5 and 5%, respectively for siblings; and 11, 17 and 21% for VUD. The overall survival at one year was in excess of 70% for both groups with a disease free survival of 61 and 59%. In addition, 86% of the recipients achieved full donor chimerism. The cumulative incidence of grade 3/4 GvHD at day 100 for VUD recipients was 9% and for sibling recipients 0%, indicating the effectiveness of CAMPATH-1 in reducing GvHD. The results were particularly encouraging in low risk MDS patients and AML patients who were in complete morphological and/or cytogenetic remission at the time of transplantation. In contrast, poor prognosis AML patients (i.e. poor risk cytogenetics at diagnosis, only partial remission or progressive leukaemia at the time of transplant) have a high risk of relapse, despite repeated DLIs, with median survival from transplantations for those with relapsed/progressive leukaemia at 68 days and those with impartial remission at 162 days. For these patients there are currently few other treatment options.

Recomposition of an anti-tumour immune response

Whilst immune editing of the tumour takes place through a strong anti-tumour response against the most immunogenic tumour antigens, the immune system also becomes simultaneously "edited" through exhaustion [63, 64]. By combining the essence of surgery, chemotherapy, HSC transplantation and immunotherapy, a window of opportunity may be created to stimulate an un-edited, or at least less edited immune system, to combat a reduced tumour mass which is more likely to be susceptible to eradiation (e.g. reduced expression of immune suppressive factors, numerically reduced chance of generating escape mutants, etc). An example of this strategy is outlined in our Phase I clinical study which combines RIC and HSCT with immune gene therapy of AML.

A Phase-I clinical study for the induction of GvL effect in poor prognosis AML

We have recently obtained Gene Therapy Advisory Committee (GTAC) approval to undertake a Phase-I study involving the immunisation of poor prognosis AML patients who have had an allogeneic HSCT, and have achieved partial or complete remission and clear evidence of donor chimerism, with their own leukaemic cells that are transduced by a self-inactivating lentiviral vector encoding B7.1 and IL-2 (Fig. 2a). This trial, which builds on the established therapeutic efficacy of allogeneic HSCT and DLI, will have a number of important and relatively unique features:

- The tumour cell vaccine naturally express both MHC class I and II, as well as a large number of adhesion and co-stimulatory molecules that are normally expressed by the professional APCs—but importantly lack innate B7.1 expression.
- The genetically modified AML cells will express the missing B7.1 in combination with IL-2. They may therefore represent effective APCs that could stimulate both helper and effector T cell functions.
- The vaccinated patients will have recently received an allogeneic HSCT and DLI, providing a relatively intact, donor derived, immune system and reduced numbers of tolerised, anergised and regulatory T cells.
- The vaccinated patients will be in early relapse, therefore, the leukaemic cell burden will be low, thus reducing the chances of "blunting" an otherwise effective immunological response, or the risk of generating escape mutants.

IL-2 a double edge sword!

The clinical application of IL-2 mediated therapies carry a number of potential side effects. The first is the well established systemic toxicity of high dose IL-2 (vascular leak syndrome). In particular, the higher relative toxicity of IL-2 in man, compared to mice, has prevented the clinical administration of IL-2 at doses that are high enough for maximal therapeutic efficacy. Therefore, many of the most promising anti-tumour effects of IL-2 in murine studies have not been reproducible in clinical trials. The second major consideration is the ability of IL-2 to stimulate the expansion and/or activity of regulatory T cells (T_{Reg}: $CD4^+$/$CD25^{Hi}$/$FoxP3^+$). IL-2 therapy can therefore impede anti-tumour immunity by activating the immune suppressive effects of T_{Reg} [65, 66]. Indeed, recent studies have shown that elimination of T_{Reg} cells (with the aid of a recombinant IL-2 diphteria toxin conjugate—Ontak) substantially enhances anti-tumour immunity in renal cell carcinoma patients that are subsequently vaccinated with tumour RNA transfected dendritic cells [67]. The IL-2 toxicity, and in

particular its effect on regulatory T cells and thus the potential abrogation of anti-tumour immunity, are both concentration dependent. The systemic dose of IL-2 at which both the vascular leak syndrome and increased numbers of T_{Regs} are detectable is in the order of about 700,000 IU (1 IU equals approx. 0.4 ng) per Kg weight, administered as bolus injections up to three times a day for several days [66]. However the in vitro concentration of IL-2 at which the expansion/activation of tumour reactive/specific T cells become clearly visible is at least a 10^3–10^4 fold lower than this (i.e. 1–20 ng/ml). Furthermore, in murine tumour models the therapeutic effect of vaccination with IL-2 secreting tumour cells are visible at IL-2 doses that are 10^3–10^4 fold lower than the levels used in systemic IL-2 administration in the treatment of renal cell carcinoma. The highest dose of administration with B7.1/IL-2 expressing AML cells in our trial will be 10^8 lethally irradiated cells per dose injected at three weekly intervals for up to a maximum of six doses. Therefore the systemic IL-2 levels in these patients are likely to be at least a 1,000 fold lower than the levels associated with either in vivo toxicity or stimulation of T_{Regs}; although the minimum IL-2 dose at which T_{Reg} stimulation is achieved has not yet been established in clinical trials [66]. Of course the local concentration of IL-2 in the vicinity of the B7.1/IL-2 expressing AML cells is likely to be substantially higher, thus resulting not only in the effective stimulation of cytotoxic T cells, but also the possible expansion/activation of the immune suppressive T_{Reg}.

In the light of these considerations, the risk of either IL-2 toxicity or the suppression of anti-leukaemic immunity due to its activation of T_{Reg} remain potential risks associated with vaccination using IL-2 expressing tumour cells. Therefore, the detailed analysis of both toxicity, and T_{Reg} proliferation/activity remain amongst the central objectives of our B7.1/IL-2 vaccination studies. Furthermore, it remains entirely conceivable that elimination of T_{Reg} will enhance the anti-leukaemic effect of HSCT and donor leukocyte infusion, either with or without concomitant vaccination with B7.1/IL-2 expressing AML cells.

Concluding remarks

The success of haematopoietic stem cell transplantation (HSCT) and donor leukocyte infusions (DLI) in the rejection of acute myeloid leukaemia, demonstrate the efficacy of the donor immune system in recognising and eradicating AML blasts. The success of this established form of immune therapy against AML, provides grounds for optimism to be able to enhance the efficacy of immune mediated rejection of AML, and in particular eradication of the minimal residual disease that is the cause of subsequent relapse in the poor prognosis patients. Given the fact that the leukaemia and the immune system are likely to have sculpted each other in ways dictated by their continued evolution in the presence of

each other, effective stimulation of immune therapy requires both the reduction of the tumour mass, and elimination of immune tolerance. The former is frequently achievable by chemotherapy, albeit for a transitory period in the poor prognosis AML, and the latter can be achieved, at least in part by HSCT and DLI. Preclinical studies showing that the B7.1/IL-2 expressing AML cells can stimulate in vitro cytolytic activity against the unmodified AML cells suggest the potential possibility of vaccination mediated enhancement of immune mediated rejection of residual AML by a donor immune system that is not exhausted by the process of tumour editing. Potential hazards with such vaccinations include the stimulation of graft versus host disease without an even greater stimulation of leukaemia rejection, IL-2 associated toxicities, and possibly even T_{Reg} mediated abrogation of HSCT and DLI associated immunity against AML. Given the established role of immune therapy in AML (HSCT and DLI), and the availability of clinically feasible strategies for the elimination of T_{Reg}, the clinical effect of T_{Reg} elimination in the treatment of AML, warrants direct evaluation.

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