Can immunotherapy specifically target acute myeloid leukemic stem cells?

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells (“blasts”) that is characterized by maturation arrest, uncontrolled proliferation, and resistance to apoptosis.1 Although 65 to 75% of AML patients younger than 60 reach a complete hematological remission in response to currently available therapies, the five year overall survival is less than 30%, owing to a high relapse rate.1 Accumulating evidence supports a prominent role for leukemic stem cells (LSCs) in such a high rate of therapeutic failures.1–6 LSCs possess biological properties that render them resistant to chemotherapy and radiotherapy and hence are probably responsible for the minimal residual disease (MRD) of AML patients, eventually leading to relapse. Therefore, the targeted elimination of LSCs may constitute a very efficient way to achieve durable remissions in the absence of prominent side effects.2,5,7

Leukemic Stem Cells: Definition

Most AML cells are unable to proliferate extensively, and only a subset of these cells preserves clonogenic properties, suggesting that, similar to normal hematopoiesis, leukemia may be maintained by a small population of stem cells.3–8 In 1994, Dick and colleagues identified, based on transplantation experiments in severe combined immunodeficient (SCID) mice, AML-initiating cells as residing within the CD34+CD38− population, similar to normal hematopoietic stem cells (HSCs). These AML-initiating cells were called leukemic stem cells (LSCs). In this model, the CD34+CD38− or CD34+ AML cell fractions were not capable of generating a leukemic graft.3 In 1997, the same group reported (in the non-obese diabetic (NOD)/SCID mouse model) that LSCs exhibited a CD34+CD38− phenotype in every patient tested, regardless of the French-American-British (FAB)
subtypes of disease (with the exception of AML-M3) and lineage markers expressed by the bulk of leukemic blasts. In addition, serial transplantation experiments performed by Shultz et al. in xenotransplant-permissive NOD/SCID/Il2rg−/− (NSG) mice, demonstrated that long-term engraftment and the self-renewal capacity of human AML cells resided exclusively in the CD34+CD38− population. This was illustrated by the maintenance of human AML for over 1 year in vivo, using serial transplants.15 LSCs were shown to be mainly in the G0 phase of the cell cycle, confirming their quiescent nature.12,13 The estimated frequency of LSCs in the different in vivo stem cell assays performed varied between $1 \times 10^{-6}$ to $1 \times 10^{-2}$ of the total leukemic population.3,8,10,12,14

Despite these studies, controversy about the immunophenotype of the LSC arose (Table 1). Taussig and colleagues stated that, when grafted into NOD/SCID mice, the CD34+CD38− fraction of certain AML samples contained all, or at least most, LSCs. However, this was evaluated by the percentage of engraftment only 6 weeks after transplantation and no serial transplants were performed. Taussig et al. explained the discrepancy between their observations and previous findings3,10 by suggesting an inhibitory effect on the engraftment of CD34+ AML cells that would have resulted from the anti-CD38 antibody used in prior studies.15 The same group demonstrated by means of serial transplantation experiments that LSCs were contained in the CD34+ fraction of 15/15 AML samples obtained from patients with nucleophosmin (NPM)-mutated disease, whereas the CD34− fraction engrafted only in half of the samples.16 Recently, Dick and colleagues reported—by means of an optimized NOD/SCID model based on intrafemoral injections—that LSCs could be detected in the CD34+CD38− fraction of each investigated case but one. However, although LSCs were enriched in the CD34+CD38− compartment, they could also be detected in the CD34−CD38− cell population in about half of the patients, and in some patients LSCs were found in the CD34− fraction, suggesting a heterogeneity of cell surface marker expression and LSC activity among individual samples.14

Despite accumulating evidence in support of the LSC-concept,3,10,11,17 there is still some controversy about whether LSCs can really be considered as stem cells. To answer this question, one needs to verify whether all minimal criteria for stem cell-ness are fulfilled. So far, the challenge of demonstrating the coexistence of both self-renewal and differentiation within a single cell remains, and therefore the LSC concept is not yet definitive.18

**Leukemic Stem Cells: Artifacts of Xenotransplantation or Clinically Relevant?**

If LSCs, as defined in mouse models, were also relevant for AML patients, they may constitute the main targets for consolidation therapy against MRD.19 Van Rhenen et al. demonstrated in 2005 that a high frequency of CD34+CD38− LSCs at AML diagnosis predicts high frequencies of MRD after chemotherapy and poor overall, disease-free and relapse-free survival, both in an in vivo model and in correlation studies in patients.6 Another study reported that the relative ability of AML cells to successfully engraft in immunodeficient mice (a property associated with LSCs) correlated with adverse clinical features.20 Recently, two groups have independently demonstrated that HSC- and LSC-enriched populations share very similar transcriptional “stem cell-like” or “self-renewal” gene expression signatures that reflect stem cell function in vivo14 and that are predictive of adverse clinical outcome in individuals with AML.14,21 The predictive value of this LSC score appeared to be independent of other risk factors in multivariate Cox regression analysis, which further supports the clinical relevance of LSCs.14,21

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**Table 1. Definition of leukemic stem cells in immunodeficient mouse models**

| Ref. | AML characteristics | Source | Immunophenotype | Type (age, mouse strain, administration) | Engraftment | Serial transplant |
|------|---------------------|--------|-----------------|----------------------------------------|-------------|------------------|
| 3    | M1/M2/M4(FAB)       | PB     | Yes Yes No No   | Adult, SCID, i.v.                       | 30–45 d     | No               |
| 10   | M1/M2/M4/M5 (FAB)   | PB     | Yes Yes No No   | Adult, NOD/SCID, i.v.                   | 4–8 wk      | Yes              |
| 12   | M1/M2/M3/M4/M7 (FAB)| BM     | Yes No No Yes   | Adult, NOD/SCID/Il2rg−/−, i.v.          | 16 wk       | Yes              |
| 15   | NPM + and −         | NA     | Yes Yes Yes Yes | NPM+, NOD/SCID and NOD/SCID/B2m−/− and NOD/SCID/Il2rg−/−, i.v. | 6 wk       | No               |
| 16   | All NPM+, M1/ M2/ M4/ M5 (FAB) | PB       | Yes NA Na Yes | Adult/NOD-SCID B2m+/- and Adult/NOD-SCID Il2rg+/- IBM and i.v. | 9–15 wk | Yes |
| 14   | NPM+ and −M1/ M2/ M4/ M5 (FAB) | PB     | Yes Yes Yes Yes | Adult, NOD/SCID, IF | 6,5–15 wk (mean 10 wk) | Yes |

**Abbreviations:** AML, acute myeloid leukemia; BM, bone marrow; FAB, French-American-British; IBM, intra-bone marrow; IF, intrafemoral; LSC, leukemic stem cell; NA, not addressed; NPM+, nucleophosmin-mutated; NPM−, nucleophosmin non-mutated; PB, peripheral blood. Source indicates the source of cells (peripheral blood or bone marrow) from AML patients used for in vivo assays. Immunophenotype addresses the question whether LSCs are contained within the specified populations (yes or no). The characteristics of the mouse model are shown in type (age, strain and administration route) and the interval between transplantation and analysis in engraftment.
In order to determine the number of LSCs surviving therapy and to design LSC-targeted therapies, many attempts have been made to refine the cytofluorometric CD34+CD38− phenotype of LSCs with markers for discriminating between LSCs and normal HSCs, which are also contained within the CD34+CD38− fraction and can be present in (especially bone marrow) patient samples. Both LSCs and HSCs are PKHbright (PKH is fluorescent dye diluting upon cell division) and aldehyde dehydrogenase (ALDH)bright.22,23 Similarly, both LSCs and HSCs are believed to reside in the so-called “side population,” which has a very efficient capacity of effluxing the chromatophytic dye Hoechst 33342.24 Aberrant cross-lineage marker expression of antigens such as CD7, CD19 and CD56 can, in some cases, suggest the malignant nature of the CD34+CD38− cells, as can do the asynchronous, decreased or increased expression of classical progenitor markers. These aberrant antigen expression sets can be determined at diagnosis, and can be useful to estimate the frequency of the MRD/LSCs more accurately after chemotherapy, but their therapeutic applications are not obvious.24,25 However, surface markers such as C-type lectin-like 1 (CLL-1/MICL/CLEC12A), CD123, CD44, CD47, CD96 and CD25 were shown to be differentially expressed in LSCs vs. HSCs. These potential (immuno)therapeutic targets will be discussed below.2,5,25–29

**Leukemic Stem Cells: Gene Expression Profile**

When comparing the gene expression profiles of the CD34+CD38− and CD34+CD38+ fractions of peripheral blood obtained from AML patients, Gal et al. found that 409 genes were differentially expressed.30 Proliferation-, cell cycle- and differentiation-related genes were shown to be systematically repressed in the LSC-enriched subpopulation, which is consistent with a tendency toward replicative quiescence.2,21 However, 138 of 409 under- or overexpressed genes in LSCs were reported to behave similarly in normal HSCs in other data sets.30 The LSC signature described by Gentles et al. consisted of 31 genes overexpressed in LSCs (and normal HSCs) as compared with non-stem cell leukemia blasts.21

Despite a considerable overlap with HSCs,14,21,30 the gene expression profiles of AML stem cells also demonstrate dysregulated gene expression networks and differentially expressed markers that could become attractive targets for LSC-specific therapies.2,26,30 Majeti et al. identified 3005 genes differentially expressed in (bone marrow or peripheral blood) CD34+CD38− LSCs vs. (bone marrow) CD34+CD38+. HSCs. The signaling pathways that appeared to be dysregulated in LSCs included molecular cascades involved in adherens junction, the actin cytoskeleton, apoptosis, MAPK signaling and WNT signaling.7

**Leukemic Stem Cells: Importance of the Microenvironment**

Similar to situation of HSCs, the microenvironment seems to be very important for the quiescence of LSCs. Homing studies have shown that human CD34+CD38− AML cells primarily home to and are preferentially retained in the endosteal osteoblast-rich region of the femur of NSG mice, where these cells remain quiescent and hence are protected from chemotherapy-induced apoptosis.12 Quiescence in this niche is probably the reason why standard poly-chemotherapy and radiotherapy may induce remission in AML patients but only rarely cure them. Both therapies may affect descendant cells that are irrelevant for the persistence of the disease, but presumably leave LSCs unperturbed. AML patients that relapse soon after first remission typically do so while manifesting a chemoresistant disease. However, those who relapse later usually manifest a chemoresistant disease. Therefore, there is a clear need for therapeutic strategies that would ideally target LSCs while sparing self-renewing normal HSCs, hence securing normal long-term hematopoeisis in patients.

**Leukemic Stem Cells: Ultimate Targets For Therapy?**

Different therapeutic strategies to target LSCs have already been explored, including the induction of proliferation with cytokines such as granulocyte-colony stimulating factor (G-CSF) to increase the responsivenss to standard therapy,32 and the impairment of their self-renewal capacity or survival by targeting—for example—the WNT/β-catenin pathway.33 In addition, strategies have been tested that isolate LSCs from the stem cell niche by interfering with ligand/receptor interactions in order to both abolish the microenvironmental protection against apoptosis and to obtain a higher proliferation rate.34,35 Finally, immunotherapy is expected to be successful in the setting of MRD, complementary to prior standard treatment. Immunotherapeutic strategies, including monoclonal antibodies (mAbs) and T cell-mediated approaches, that target leukemia-associated antigens (LAAs) expressed by LSCs, will be discussed below in more detail.

**Immunotherapy: Therapeutic Monoclonal Antibodies**

mAbs, usually in combination with chemotherapy, have been demonstrated to constitute effective targeted therapies for the treatment of a number of hematological and non-hematological malignancies. Given their antigen-specificity and minimal toxicity, they may be an excellent LSC-targeting therapy. This immunotherapeutic strategy is hypothesized to function through several mechanisms: antibody-dependent cellular cytotoxicity, complement activation, a direct pro-apoptotic effect as well as upon the inhibition of signal transduction cascades that are essential for homeostasis, proliferation or interaction with the microenvironment.36 Finally, mAbs can be conjugated to radioisotopes or toxins. So far, a large number of studies have aimed to identify candidate cell surface targets that are specifically or preferentially expressed on LSCs and not—or less so—on normal HSCs and other vital tissues (Tables 2 and 3).

However, multiple leukemia-associated antigens are widely expressed on normal tissues, albeit sometimes at comparatively lower levels. CD44, also known as homing-associated adhesion...
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Expression was also detected in about 5% of normal HSCs and in multiple non-hematopoietic tissues. 29,39 Monoclonal antibodies targeting CD44 or CD47 but not CD96 have been tested in NOD/SCID and newborn NOD/Shi-SCID/Il2rg−/− (NOG) mice, and showed promising results against AML cells including LSCs, 5,28 but potential toxic effects due to unspecific target expression could not be evaluated in these models. No clinical trials have been conducted using mAbs directed against CD44, CD47 or CD96, but considering their unspecific expression pattern, important hematological and non-hematological toxicity may be expected.

Table 2. Expression of leukemia-associated antigens in AML, leukemic stem cells, normal tissues and hematopoietic stem cells

| Antigen            | Specifications | % AML* | % LSC | LSC/bulk | LSC | HSC | Normal tissues other than HSC |
|--------------------|---------------|--------|-------|----------|-----|-----|-------------------------------|
| AURKA              | Aurora kinase A | 37±3   | NA    | NA       | ++7,26, ++ (CML)39 | + (BM/CB)30 | + Ubiquitous during mitosis69 |
| CD25               | IL-2 receptor α | NA     | 25±6  | NA       | + (25%)36 | − (BM)28 | + Hematopoietic cells, ++ activated T cells and Tregs20,41 |
| CD33               | Siglec-3      | > 80±6 | 100±7 | 1±6      | ++++, M80%27,44 | ++++, m84% (BM); ++++, m > 80% (RBM)22; +, m > 80% (CB/BM)49; ++ (BM)45 | + Normal HPCs65 |
| CD44               | H-CAM         | 100±7  | 100±7 | < 1±     | ++5 | + (CB)2; + (BM)7 | + Ubiquitous, activated T cells2,3±31 |
| CD47               | Integrin associated protein Ig superfamily | 100; high: 25±18 | 100±18 | 1±28 | +,2,28,38 | (BM)28,38 | + Ubiquitous28 |
| CD96               | Tactile, Ig superfamily | 30±29 | 66±25 | NA | ++++, m74%27,29 | +,m5% (BM)27 | ++ Activated T cells, activated NK cells, lung, spleen, thymus; + ubiquitous16,41 |
| CD123              | IL-3 receptor α | 100±22 | 100±22 | NA | ++++,m90%5,82 | − (BM)22; +, M27% (BM)34; +, M20/60% (CB/BM)44; ++, M60% (RBM)45 | + Normal BM: 7%±2 |
| CLL-1              | C-type lectin-like molecule | 92±7 | 87±27 | NA | ++, M33%27 | − (BM, RBM, MPB)35,27 | + CD34+CD38+ HPCs, monocytes, dendritic cells, granulocytes27,47 |
| MUC1               | Mucin 1       | 67–70±64,84 | NA | 1±22 | ++ (CD34)+5,28 | − (BM, RBM)44; ☐ (BM)25 | + HPCs, secretory epithelial cells, B cells, activated T cells44,64 |
| RHAMM              | Receptor for hyaluron mediated motility | 60–70±3 | 0±3 | < 1±5,63 | −/−,20,63 | − (CB)63 | ++ Activated T cells, engrafting HPC (CD341), thymus, testis, placenta; + colonic and gastric mucosa, cornea/limbus69 |
| WT1                | Wilms’ tumor 1 | 73–100±71 | 73–100±71 | 1±20 | ++,1,28,26,71 | +/+/ (CB, BM,MPB)71,73 | + HPCs, podocytes of glomerulus, Sertoli cells testis, granulosa cells ovary, mesothelium, mammary duct and lobule71 |

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; CB, cord blood; HPC, hematopoietic progenitor; Ig, immunoglobulin; IL, interleukin; LSC, leukemic stem cell; MPB, mobilized peripheral blood; NA, not addressed; NK, natural killer; PB, peripheral blood; RBM, regenerating bone marrow; Treg, regulatory T cell. *% AML, % of positive bulk AML patient samples; % LSC, % of positive LSC patient samples; LSC/bulk: ratio of expression levels between LSCs and bulk AML cells from the same patient. LSC and HSC: % values indicate the median (M) or mean (m) percentage of positive cells within the leukemic and hematopoietic stem cell samples. Normal tissues other than HSC indicates the expression level in normal tissues. Expression level: −, no expression; +, low expression; ++, moderate expression; ++++, high expression.
Table 3. Efficacy and toxicity of immunotherapeutic strategies targeting leukemia-associated antigens

| Antigen | In vitro efficacy on bulk AML cells or LSCs* | In vitro toxicity | Preclinical efficacy in vivo on AML cells or LSCs | Preclinical toxicity in vivo | Clinical efficacy | Clinical toxicity |
|---------|------------------------------------------------|------------------|------------------------------------------------|-----------------------------|-------------------|-------------------|
| CD33    | GO: yes/yes36                                   | No (HSC)46       | Yes (HL-60 cell line)/NA45                       | Yes: mouse (human CB HSC engraftment)44 | II: GO: relapsed AML: yes (OR 26%)*4| II/III: GO: no serial a.e.45,46 |
|         |                                                |                  |                                                 | GO: yes: monkey/rat (liver toxicity)45 | II: GO: post-remission AML: no*4| II/III: low-dose GO: less liver toxicity*45 |
| CD44    | Yes/yes41                                       | Yes (myelopoiesis)41 | Yes/yes5 | Yes: mouse (human CB engraftment)5 | NA                  | NA                |
| CD47    | NA/yes26                                       | No (CD34 BM)28    | Yes/yes28 | No: mouse28 | NA                  | NA                |
| CD123   | Yes/yes27                                      | NA               | Yes/yes2 | CSL60: no: monkey2 | I: CSL60: relapsed/refractory/HR AML: yes (1 /26 CR)44 | II: peptide: relapsed/refractory AML: yes*4 |
| WT1     | Yes (cell lines)/yes (CML)74,85                 | No (CD34 BM,CB,MBP)73 | Yes/yes2 | CSL60: no: monkey2 | I: CSL60: no: no serious a.e.48 | II: peptide: no: no serious a.e.75 |

Abbreviations, I/II/III, Phase I/II/III clinical trial; a.e., adverse events; AML, acute myeloid leukemia; BM, bone marrow; CB, cord blood; CML, chronic myeloid leukemia; CR, complete remission; DC, dendritic cell; GO, gemtuzumab ozogamicin; HR, high risk; LSC, leukemic stem cell; MR, molecular remission; NA, not addressed; OR, overall response rate; PR, partial remission; PR > CR, PR prior to treatment is turned into CR. *In vitro efficacy on AML cells or LSCs indicates whether in vitro experiments have demonstrated an effect on the bulk of AML cells or LSCs in particular. In vitro toxicity and preclinical toxicity in vivo indicates whether toxicities have been reported in vitro and in vivo in animal models, respectively. Preclinical efficacy in vivo on AML cells or LSCs indicates whether xenograft mouse models have demonstrated an effect on the bulk of AML cells or LSCs in particular. Clinical efficacy and clinical toxicity illustrate the efficacy and toxicity reported in Phase I/II/III clinical trials.

Moreover, some potential antigens for the development of LSC-targeting approaches are upregulated in normal tissues under non-homeostatic conditions, such as during immune activation or in the regenerating bone marrow. CD47 serves as a ligand for signal regulatory protein α (SIRPα), which is expressed on macrophages and dendritic cells. Upon the binding of CD47 to SIRPα, the phagocytic activity of these cells is inhibited. Therefore, Jaiswal et al. suggested that CD47-SIRPα interaction is one of the mechanisms used by AML cells to escape the innate immune system. However, CD47 expression was shown to be a more universal mechanism of ‘self’ protection against phagocytosis, as illustrated by a transient upregulation on mouse HSCs upon mobilization to the peripheral blood. Both CD44 and CD96 were shown to be strongly upregulated in activated T cells, compared with resting T cells. CD25, the α chain of the interleukin-2 (IL-2) receptor, was shown to be expressed by LSCs in 24.6% of AML samples, yet is also expressed by activated T cells and regulatory T cells (Tregs). Clinical experience with daclizumab, an anti-CD25 mAb, was obtained in strategies for the prevention of organ allograft rejection, demonstrating its immunosuppressive features. Therefore, targeting CD96, CD44 or CD25 might result in the depletion of activated T cells, which could increase susceptibility to infections and could hamper antibody-dependent cell-mediated cytotoxicity. On the other hand, the neutralization of Tregs by anti-CD25 mAbs may revert the immunological anergy against AML.

The expression of CD123 (the IL-3 receptor α chain) was detected in a median percentage of 60% of the normal CD34+/CD38− cells in the bone marrow of AML patients who were in remission and recovering from chemotherapy. This indicates that the expression pattern of CD123 might be influenced by external factors, and that targeting CD123 might hamper the recovery of normal hematopoiesis. CD33 expression was demonstrated on the CD34+CD38− fraction of a majority of AML cells or LSCs in particular. In vitro and preclinical toxicity in vivo indicates whether toxicities have been reported in vitro and in vivo in animal models, respectively. Preclinical efficacy in vivo on AML cells or LSCs indicates whether xenograft mouse models have demonstrated an effect on the bulk of AML cells or LSCs in particular. Clinical efficacy and clinical toxicity illustrate the efficacy and toxicity reported in Phase I/II/III clinical trials.
The success of allogeneic hematopoietic stem cell transplantation (allo-HSCT) in curing AML has largely been attributed to the graft vs. leukemia (GvL) effect, especially in the context of non-myoeloblastic HSCs. In particular, donor lymphocyte infusions (DLIs) have been shown to constitute an effective salvage therapy for up to 40% of AML patients who relapsed after HSCT, supporting the essential role for donor T cells in eradicating residual leukemic (stem) cells. More specifically, the role of minor histocompatibility antigen (MIHA)-specific T cells in GvL reactivity has been demonstrated by the expansion of HA-1- and HA-2-specific T cells that preceded clinical responses after allo-HSCT and has been demonstrated by the expansion of HA-1- and HA-2-specific leukemic antigen (MIHA)-specific T cells in GvL reactivity results are promising, with an acceptable safety profile. Mainly because of severe hepatic toxicity that became evident in post-marketing studies, the drug was withdrawn from the market in 2010. Subsequently, dosing and scheduling changes were proposed in an attempt to improve feasibility. Currently, a Phase II/III clinical trial (NCT00091234) is ongoing that uses reduced doses of gemtuzumab ozogamicin as induction monotherapy in older patients unfit for conventional chemotherapy. Mainly because of severe hepatic toxicity that became evident in post-marketing studies, the drug was withdrawn from the market in 2010. Subsequently, dosing and scheduling changes were proposed in an attempt to improve feasibility. Currently, a Phase II/III clinical trial (NCT00091234) is ongoing that uses reduced doses of gemtuzumab ozogamicin as induction monotherapy in older patients unfit for conventional chemotherapy. The first results are promising, with an acceptable safety profile.

### Immunotherapy: T Cell-Mediated Therapies

The success of allogeneic hematopoietic stem cell transplantation (allo-HSCT) in curing AML has largely been attributed to the graft vs. leukemia (GvL) effect, especially in the context of non-myoeloblastic HSCs. In particular, donor lymphocyte infusions (DLIs) have been shown to constitute an effective salvage therapy for up to 40% of AML patients who relapsed after HSCT, supporting the essential role for donor T cells in eradicating residual leukemic (stem) cells. More specifically, the role of minor histocompatibility antigen (MIHA)-specific T cells in GvL reactivity has been demonstrated by the expansion of HA-1- and HA-2-specific T cells that preceded clinical responses after allo-HSCT and subsequent DLI. In the past decade, there have been a lot of efforts to characterize dominant LAAs recognized by T cells that might also contribute to the GvL effect. (Tables 2 and 3). This may allow the development of a specific T-cell based immunotherapy, not only in the context of allo-HSCT and DLI, but also for patients who are not eligible for allo-HSCT because of their age, comorbidities or due to donor unavailability.

Both active strategies—such as peptide, DNA or dendritic cell-based vaccinations—and passive strategies—such as the adoptive transfer of AML-specific T cells—can be used in order to stimulate the patient’s cytotoxic capacity against AML cells. However, most LAAs are self-antigens for which it is difficult to generate high affinity T-cell responses in vivo in an autologous setting. The passive transfer of autologous or donor T cells genetically modified to express a high affinity LAA-specific T cell receptor (TCR) is therefore more likely to induce potent immune responses. Such a TCR can be isolated from LAA-specific T cells generated in vivo or in vitro in an allogeneic major histocompatibility complex (MHC)-mismatched setting.

A priority-ranked list of cancer vaccine target antigens was published in 2009 by the Translational Research Working Group of the National Cancer Institute (NCI), ranking cancer-associated antigens based on predefined and pre-weighted objective criteria determining the likelihood of efficacy in cancer therapy. These criteria included therapeutic function in vaccine trials, immunogenicity, the number of patients with antigen-positive tumors, expression level, the percentage of positive tumor cells and cellular location, a role for the antigen in oncogenicity, as well as a tumor-specific expression profile and expression in cancer stem cells. In the last decade, the latter criterion has become a weighty issue for AML-directed T cell-mediated immunotherapy.

Some LAAs are highly expressed by the majority of bulk AML samples, but the expression by LSCs may be significantly lower or even undetectable. In particular, antigens associated with proliferation are expected to be downregulated in dormant LSCs. We and others found that receptor for hyaluronic acid mediated motility (RHAMM) expression is significantly lower in CD34+ CD38- AML cells than in the comparatively more mature CD34+ CD38+ fraction, indicating that LSCs may not be targeted by RHAMM-directed immunotherapy. Although listed within the top 75 of prioritized antigens, cyclin B1, cytochrome P450 1B1 (CYP1B1), proteinase 3 and survivin exhibited a similar trend toward under-expression in LSCs. On the expression of mucin 1 (MUC1), the second hit in the NCI ranking, by LSCs are controversial.

Other LAAs, such as B lymphoma Mo-MLV insertion region 1 homolog (BMI-1), are equally expressed by LSCs and CD34+ CD38+ cells, but are also expressed at similar levels by normal HSCs. A comparable expression is especially found if antigens, like BMI-1, are involved in self-renewal or quiescence, which are shared characteristics of HSCs and LSCs. Approximately 10% of cord blood CD34+ cells express MUC1. Fatrai et al. even demonstrated that MUC1 mRNA expression was the highest in the CD34+ CD38+ fraction of cord blood as compared with more differentiated normal progenitor subsets. Cyclin B1 expression levels seem to be lower in LSCs than in normal HSCs.

Targeting LAAs may result in important toxicities if these antigens are highly expressed by HSCs or by normal tissues under non-homeostatic conditions such as T-cell activation, tissue regeneration and hematopoietic recovery after chemotherapy or allo-HSCT. We showed that RHAMM, being a proliferation marker, is upregulated during the engraftment of human HSCs in NOD/SCID mice and upon T-cell activation. Similarly, an increased expression of survivin and MUC1 was observed in activated T cells.
With regard to survivin, this upregulation resulted in the fratricide of activated T cells by survivin-specific T cells.66 Likewise, Aurora kinase A (AURKA), a serine/threonine kinase that is required for the assembly of the mitotic spindle, has been shown to be expressed at low levels by normal cells, in particular in dividing cells and in testis.67,68 In vitro, no lysis of resting cord blood CD34+ cells was seen,67,69 but proliferation during engraftment may lead to the upregulation of AURKA and potential lysis by AURKA-specific T cells. Somewhat reassuring was the fact that activated (proliferating) T cells showed only a small increase in AURKA mRNA expression and were not lysed in vitro by AURKA-specific T cells.69 Anti-leukemic activity was demonstrated for both AURKA- and RHAMM-specific T cells in vivo, but toxicity due to non-specific expression cannot be evaluated in xenograft models.

Wilms’ tumor 1 (WT1), a zinc finger transcription factor, was the antigen with the highest cumulative score (0.81/1) in the NCI ranking of cancer antigens and WT1-based immunotherapeutic approaches are advancing in (pre)clinical development.62 WT1, which is overexpressed in 73–100% of AML patients, was identified as a promising candidate for LSC targeting, since the gene was overrepresented in the majority of LSC samples at levels that are similar to those of comparatively more mature AML cells.7,26,73 Moreover, WT1 expression has been demonstrated in quiescent cells lining the endostium of the bone marrow in a NSG xenograft model.74 After pretreatment with WT1-specific T cells, a virtually complete inhibition of the engraftment of CD34+ cells from chronic myeloid leukemia (CML) patients in NOD/SCID mice was reported, suggesting a bona fide targeting of LSCs. No data are available yet relative to AML LSCs.

Unfortunately, WT1 expression has also been detected in normal HSCs, even though at comparatively much lower levels.7,26,73 Anti-WT1 cytotoxic T-cell clones that were able to eliminate CML cells in vitro did not inhibit colony formation of normal CD34+ cells. This indicates that HSCs do not express sufficient levels of WT1 to trigger a cytotoxic T-cell attack.73 WT1 is also expressed in the glomerular podocytes, Sertoli cells of the testes, ovarian granulosa cells, mesothelial cells and mammary ducts and lobules, increasing the risk for unwanted toxicities.71 In Phase I/II clinical trials (NCT00153582, NCT00834002) testing peptide and dendritic cell-based vaccinations, no adverse events related to unspecific expression, such as renal toxicity or profound cytopenias, could be observed.74,75 The specific targeting of LSCs in vivo has not yet been investigated and overall clinical responses in vaccination trials were rather modest up to now.74,75 Multiple Phase I/II studies (NCT01640301, NCT01621724) involving the adoptive transfer of donor or autologous T cells transduced with a WT1-specific TCR in patients with AML are ongoing. It is not clear whether these more potent adoptive T cell-based strategies will induce adverse effects due to unspecific cell killing.

**Future Perspectives**

In order to overcome the imminent immune tolerance toward AML cells, which express mainly self antigens, a thoughtful and personalized strategy that integrates the promising potency of immunotherapy into the current standard treatment is necessary. The quest for the ideal immunogenic AML antigen, being expressed by relapse-provoking LSCs but not by normal hematopoietic cells nor by other vital physiological tissues, has been ongoing for many years. Major or minor shortcomings have been identified for every known AML antigen, with WT1 being viewed as the closest to an ideal antigen so far.62

Some AML-specific translocations like t(8;21) and t(6;9) give rise to fusion proteins (AML1-ETO and DEK-CAN, respectively). These fusion proteins and other mutated proteins such as mutated NPM1 and FLT3 can be immunogenic62 and, as they are both genuine AML-specific targets and expressed in LSCs,77,78 they are very attractive targets for immunotherapy.62,76,79 However, due to the large diversity of mutations among AML patients, it is not clear whether strategies targeting fusion or mutated proteins are feasible.

Recently, knowledge about the genetic expression profiles of AML cells and LSCs has increased exponentially, but mRNA expression does not always result in protein expression or peptide presentation. Mainly due to the lack of suitable detection methods, the cell surface proteome of AML cells, and more specifically AML LSCs, is still largely unknown. An extensive and quantitative analysis of the LSC surface protein landscape by mass spectrometry will presumably allow for the identification of new targets for mAbs.80 Along similar lines, appropriate targets for T cell-mediated immunotherapy can be identified by eluting and analyzing by mass spectrometry the peptides that are presented on MHC molecules by LSCs.

As observed in mouse models,2,5,28 the efficacy of immunotherapy against established full-blown disease is limited. Therefore, it will be important to fine-tune the indication of immunotherapy toward an adjuvant setting to prevent relapse. Up to now, clinical trials have been mainly conducted in patients with very low effector:target cell ratios in vivo and in the context of the immunosuppressive environment that results from large tumor burdens. As a consequence, these trials were very unlikely to report major successes. Because LSC-targeting approaches are an adjunct to conventional therapy, LSC markers must be stably expressed, even after chemotherapy.

In view of the rather modest clinical benefit of immunotherapy so far reported by clinical studies, it might be necessary to apply adapted strategies or increase the dosing of available therapeutic tools, resulting in more powerful lytic activity. This raises questions about the safety of such potent approaches, because the therapeutic window of immunotherapy is correlated with the specificity of targeted antigens. Therefore, the specificity of LAAs will have to be monitored very closely in the future. In Table 4, we propose refined specificity criteria for LAAs, and we believe that these are complementary to the NCI criteria formulated in 2009.62

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42. Nashan B, Wilde S, Spranger S, Milesovic S, Frankenberg B, Udkow W, et al. MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors. J Clin Invest 2010; 120:3869-77; PMID:20978348; http://dx.doi.org/10.1172/JCI43437.

43. Nagai R, Ochi T, Fujisawa H, An J, Shirakata T, Mineno J, et al. Aurora kinase A-specific T-cell receptor gene transfer redirects T lymphocytes to display effective antileukemia reactivity. Blood 2012; 119:368-76; PMID:22052529; http://dx.doi.org/10.1182/blood-2011-06-360535.

44. Tausig DC, Pearce DJ, Simpson C, Rohatiner AZ, Nashan B, Light S, Hardie IR, Lin A, Johnson JR; Amadori S, Suciu S, Selleslag D, Stasi R, Alimena G, Baron F, Storb R. Allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning as treatment for hematologic malignancies and inherited blood disorders. Mol Ther 2006; 13:26-41; PMID:16236202; http://dx.doi.org/10.1038/jmhef.2005.9.011.

45. Kolb HJ, Scharenberg A, Goldman JM, Hertenstein B, Jacobsen N, Acceve W, et al.; European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood 1995; 86:2041-4; PMID:7665035.

46. Weiden PL, Flourny N, Thomas ED, Prentice R, Fefer A, Buckner CD, et al. Antileukemic effect of graft-versus-host disease in human recipients of allo- genetic-marrow grafts. N Engl J Med 1979; 300:1068- 73; PMID:343792; http://dx.doi.org/10.1056/ NEJM197905103001902.

47. Weiden PL, Sullivan KM, Flourny N, Storb R, Thomas ED. Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. N Engl J Med 1981; 304:5299-33; PMID:7015133; http://dx.doi.org/10.1056/NEJM1981061830042507.

48. Mazit JW, Heemskerk MH, Kloosterboer FM, Goumyl E, Kester MG, van de Hoorn MA, et al. Hematopoietic-resistance minor histocompatibility antigens HA-1- and HA-2-specific T cells can induce complete remissions of relapsed leukemia. [Eng.]. Proc Natl Acad Sci U S A 2003; 100:2724-7; PMID:12601144; http://dx.doi.org/10.1073/pnas.0509150100.

49. Kloosterboer FM, van Luxemburg-Heijs SA, van Soest RA, Baribui AM, van Egmond HM, Strijbosch MP, et al. Direct cloning of lymphocyte-reactive T cells from patients treated with donor lymphocyte infusion shows a relative dominance of hematopoietic-resistant minor histocompatibility antigen HA-1 and HA-2 specific T cells. Leukemia 2004; 18:7988-8009; PMID:14973499; http://dx.doi.org/10.1038/sj.leu.2402397.

50. Kapp M, Stevanovic S, Fick K, Tan SM, Loefller J, Opitz A, et al. CD8+ T-cell responses to tumor-associated antigens correlate with superior relapse-free survival after allo-SCT. Bone Marrow Transplant 2009; 43:399-410; PMID:19139738; http://dx.doi.org/10.1038/bmt.2008.426.

51. Rusakiewicz S, Molidrem JJ. Immunotherapeutic peptide-vaccination with leukemia-associated antigens. Curr Opin Immunol 2006; 18:595-609; PMID:16870414; http://dx.doi.org/10.1016/j.coi.2006.07.005.

52. Ramírez F, Gao L, Fujiwara H, Suemori K, Azuma T, Yakushijin Y, Hao T, et al.; Aurora-A kinase: a novel target of cellular immunotherapy for leukemia. Blood 2009; 113:66-74; PMID:18820130; http://dx.doi.org/10.1182/blood-2008-06-166489.

53. Koll HJ, Scharenberg A, Goldman JM, Hertenstein B, Jacobsen N, Acceve W, et al.; European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood 1995; 86:2041-4; PMID:7665035.

54. Weiden PL, Flourny N, Thomas ED, Prentice R, Fefer A, Buckner CD, et al. Antileukemic effect of graft-versus-host disease in human recipients of allo- genetic-marrow grafts. N Engl J Med 1979; 300:1068- 73; PMID:343792; http://dx.doi.org/10.1056/ NEJM197905103001902.

55. Weiden PL, Sullivan KM, Flourny N, Storb R, Thomas ED. Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. N Engl J Med 1981; 304:5299-33; PMID:7015133; http://dx.doi.org/10.1056/NEJM1981061830042507.

56. Marijt WA, Heemskerk MH, Kloosterboer FM, Goumyl E, Kester MG, van de Hoorn MA, et al. Hematopoietic-resistance minor histocompatibility antigens HA-1- and HA-2-specific T cells can induce complete remissions of relapsed leukemia. [Eng.]. Proc Natl Acad Sci U S A 2003; 100:2724-7; PMID:12601144; http://dx.doi.org/10.1073/pnas.0509150100.

57. Kloosterboer FM, van Luxemburg-Heijs SA, van Soest RA, Baribui AM, van Egmond HM, Strijbosch MP, et al. Direct cloning of lymphocyte-reactive T cells from patients treated with donor lymphocyte infusion shows a relative dominance of hematopoietic-resistant minor histocompatibility antigen HA-1 and HA-2 specific T cells. Leukemia 2004; 18:7988-8009; PMID:14973499; http://dx.doi.org/10.1038/sj.leu.2402397.

58. Amadori S, Suciu S, Selleslag D, Stasi R, Alimena G, Baron F, Storb R. Allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning as treatment for hematologic malignancies and inherited blood disorders. Mol Ther 2006; 13:26-41; PMID:16236202; http://dx.doi.org/10.1038/jmhef.2005.9.011.

59. Kolb HJ, Scharenberg A, Goldman JM, Hertenstein B, Jacobsen N, Acceve W, et al.; European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood 1995; 86:2041-4; PMID:7665035.

60. Weiden PL, Flourny N, Thomas ED, Prentice R, Fefer A, Buckner CD, et al. Antileukemic effect of graft-versus-host disease in human recipients of allo- genetic-marrow grafts. N Engl J Med 1979; 300:1068- 73; PMID:343792; http://dx.doi.org/10.1056/ NEJM197905103001902.

61. Amadori S, Suciu S, Selleslag D, Stasi R, Alimena G, Baron F, Storb R. Allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning as treatment for hematologic malignancies and inherited blood disorders. Mol Ther 2006; 13:26-41; PMID:16236202; http://dx.doi.org/10.1038/jmhef.2005.9.011.

62. Nashan B, Wilde S, Spranger S, Milesovic S, Frankenberg B, Udkow W, et al. MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors. J Clin Invest 2010; 120:3869-77; PMID:20978348; http://dx.doi.org/10.1172/JCI43437.

63. Nagai R, Ochi T, Fujisawa H, An J, Shirakata T, Mineno J, et al. Aurora kinase A-specific T-cell receptor gene transfer redirects T lymphocytes to display effective antileukemia reactivity. Blood 2012; 119:368-76; PMID:22052529; http://dx.doi.org/10.1182/blood-2011-06-360535.

64. Tausig DC, Pearce DJ, Simpson C, Rohatiner AZ, Nashan B, Light S, Hardie IR, Lin A, Johnson JR; Amadori S, Suciu S, Selleslag D, Stasi R, Alimena G, Baron F, Storb R. Allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning as treatment for hematologic malignancies and inherited blood disorders. Mol Ther 2006; 13:26-41; PMID:16236202; http://dx.doi.org/10.1038/jmhef.2005.9.011.
82. Jordan CT, Upchurch D, Szilvassy SJ, Guzman ML, Howard DS, Pettigrew AL, et al. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. Leukemia 2000; 14:1777-84; PMID:11021753; http://dx.doi.org/10.1038/sj.leu.2401903.

83. Walshy E, Walsh V, Pepper C, Burnett A, Mills K. Effects of the aurora kinase inhibitors AZD1152-HQPA and ZM447439 on growth arrest and polyploidy in acute myeloid leukemia cell lines and primary blasts. [eng.]. Haematologica 2008; 93:662-9; PMID:18367484; http://dx.doi.org/10.3324/haematol.12148.

84. Brossart P, Schneider A, Dill P, Schammann T, Grünebach F, Wirths S, et al. The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. Cancer Res 2001; 61:6846-50; PMID:11559560.