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Ex vivo expansion of hematopoietic cells and their clinical use

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Abstract

Background and Objective. **Hematopoietic stem cells are being increasingly used for treatment of malignant and nonmalignant disorders. Various attempts have been made in recent years to expand and manipulate these cells in order to increase their therapeutic potential. A *Working Group on Hematopoietic Cells* has analyzed the most recent advances in this field.**

Evidence and Information Sources. **The method used for preparing this review was an informal consensus development. Members of the Working Group met three times, and the participants at these meetings examined a list of problems previously prepared by the chairman. They discussed the single points in order to achieve an agreement on different judgments, and eventually approved the final manuscript. Some authors of the present review have been working in the field of stem cell biology, processing and transplantation, and have contributed original papers in peer-reviewed journals. In addition, the material examined in the present review includes articles and abstracts published in journals covered by the Science Citation Index® and Medline®.**

State of Art. **Over the last decade, recombinant DNA technology has allowed the large scale production of cytokines controlling the proliferation and differentiation of hemo-lymphopoietic cells. Thus, in principle, *ex vivo* manipulation of hemopoiesis has become feasible. The present review covers three major area of interest in experimental and clinical hematology: manipulation of hematopoietic stem/progenitor cells, cytotoxic effector cells and antigen presenting dendritic cells. Preliminary data demonstrate the possibility of using, in a clinical setting, *ex vivo* expanded hematopoietic cells with the aim of reducing, and perhaps abrogating, the myelosuppression after high-dose chemotherapy. Concurrently, other important potential applications for *ex vivo* manipulation of hematopoietic cells have recently been investigated such as the generation and expansion of cytotoxic cells for cancer immunotherapy, and the large scale production of professional antigen presenting cells capable of initiating the process of immune response.**

Conclusions and Perspectives. **Present and future challenges in this field are represented by the expansion of true human *stem cells* without maturation, to extend this strategy to allogeneic stem cell transplantation as well as the manipulation of cycling of primitive progenitors for gene therapy programs. The selective outgrowth of normal progenitor cells over neoplastic cells to achieve tumor-free autografts may ameliorate the results of autologous transplantation. The selective production of cellular subsets to manipulate the graft versus-host and graft versus-tumor effects and anti-tumor vaccination strategies may be important to improve cellular adoptive immunotherapy.**
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Key words: hematopoietic stem cells, bone marrow, cord blood, dendritic cells, peripheral blood, allogeneic transplantation

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Hematopoietic stem cells

Following the discovery that bone marrow transplantation could be used to rescue irradiated mice, the identification and characterization of the hematopoietic stem cell has become essential in order to achieve new developments in stem cell expansion and transplantation (SCT).[1] The potential for using stem cells as vehicles for gene therapy has further increased the efforts of a number of research groups working on stem cell identification, characterization, cloning and manipulation.[2]

Self-renewal and differentiation

Marrow and blood hematopoietic cells are heterogeneous and belong to different lineages at different stages of maturity. The structural and functional integrity of the hematopoietic system is maintained by stem cells that, by definition, comprise a relatively small cell population, located mainly in the bone marrow, which can (i) undergo self-renewal to

produce stem cells or (ii) differentiate to produce progeny which is progressively unable to self-renew, irreversibly committed to one or other of the various hematopoietic lineages, and able to generate clones of up to 10^5 lineage-restricted cells that mature into specialized cells.[3] Although in recent years, (i) the development of *in vitro* and *in vivo* assays for hematopoiesis, (ii) the identification and characterization of hematopoietic growth factors, and (iii) the development of strategies for enriching hematopoietic cells have expanded our knowledge and understanding of hematopoiesis, the definition of the stem cell, originally proposed by Lajtha[4] and McCulloch,[5] has not substantially changed.

In addition to self-renewal and differentiation, a number of properties are ascribed to hematopoietic stem cells, including a high migratory potential, the ability to undergo asymmetric cell divisions, the capacity to exist in a mitotically quiescent form and extensively regenerate the different cell types that constitute the tissue in which they exist.[6]

The issues of asymmetrical and symmetrical cell divisions and the regulation of self-renewal/differentiation process are crucial when analyzing stem cell behavior and the potential for stem cell manipulation. Asymmetric cell divisions produce one differentiated daughter (progenitor cell) and another daughter that is still a stem cell (Figure 1A). When all cell divisions are necessarily asymmetric and controlled by cell-intrinsic mechanisms, no amplification of the stem cell size is possible.[7] Asymmetric divisions are referred to unequally distributed transcription factors in daughter cells,[8,9] and have been shown to be possible in hematopoietic progenitors by clone-splitting experiments.[10] Symmetric cell divisions produce either two progenitor cells or two stem cells according to a 0.5 probability of self-renewing versus differentiative divisions (Figure 1B). In this case, it can be assumed that the size of the stem cell pool can be modified by factors affecting the 0.5 probability value, i.e., factors that control the probability of self-renewing versus differentiative divisions.[7] A third model postulates that individual cell divisions can be, but not necessarily are, asymmetric with respect to daughter cell fate (Figure 1C). This model also implies that daughters behave differently due to different local environments. Although it is not known whether a single cell can switch from an asymmetric to a symmetric mode of cell division, available evidence in the hematopoietic stem cell system favors a predominance of symmetric cell divisions.

The decision of a stem cell to either self-renew or differentiate as well as the selection of a specific differentiation lineage by a multipotent progenitor during commitment have been proposed to be regulated according to either stochastic or deterministic (inductive) models. Based on computer simulation and the distributions of colony-forming units in spleen (CFU-S) in individual spleen colonies, stochastic models postulate that the decision of a stem cell to self-renew (*birth*) or to differentiate (*death*) is randomly regulated by a probability parameter "*p*" which is equal to 0.5 in steady-state conditions.[11] Deterministic models postulate the existence of lineage-specific anatomic niches that direct the differentiation of uncommitted progenitors.[12] There is experimental evidence suggesting that the hematopoietic system may employ both stochastic and deterministic strategies, probably depending upon the stage of lineage differentiation.

Based on a number of studies performed in the last three decades, the regulation of self-renewal, commitment, proliferation, maturation, and survival can be assumed to reflect highly integrated processes under control of extracellular mechanisms, including regulatory molecules and microenvironment, as well as intracellular mechanisms, including protooncogenes, cell cycle regulators, tumor suppressor genes, transcription factors. Regulatory molecules include positive (growth factors) and negative (interferons, TGF-β, MIP-1α) factors which interact in complex ways (synergism, recruitment, antagonism).[13,14] Mol-

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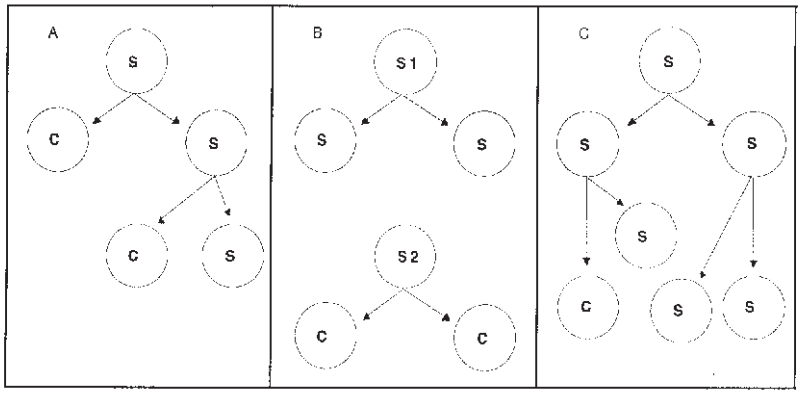

Figure 1. Possible patterns of stem cell division. "S" indicates a stem cell; "C" indicates a committed progenitor cell.

ecules that maintain the stem cell state are beginning to be identified. These include ligands of the Notch family receptors that act from outside the cell as regulators of proliferation or maintenance of the undifferentiated state,[15] and factors like PIE-1 that act from within the cell.[16] However, despite the efforts which have been devoted to elucidating the issue of self-renewal control, no factors have yet been identified that are capable of maintaining self-renewing divisions and the molecular basis of self-renewal capacity remains to be elucidated. Growth factors so far identified more probably act as regulators of proliferation and survival. Theoretically, growth factors and cell-cell interactions can influence the outcome of fate decisions by stem and progenitor cells in a selective or instructive manner. According to *selective mechanisms*, the stem cell commits to a particular lineage independently of the growth factors and the factors act to control survival and proliferation of committed progenitors. In *instructive mechanisms*, growth factors cause the stem cell to choose one lineage at the expense of others. The relative contribution of these two mechanisms to hematopoietic regulation remains controversial, but experimental evidence suggests that at least some subsets of stem/progenitor cells can be instructed by growth factors to choose one differentiation pathway at the expense of others.[17] In the absence of still unidentified instructive signals, it can be hypothesized that environmental signals may act by increasing or decreasing the probability of choosing a particular fate, rather than promoting or repressing it in an all-or-none manner.[2]

Although growth factors play a key role in stem/progenitor cell proliferation and differentiation it seems improbable that hematopoiesis is regulated by a random mix of growth factors and responsive cells. Indeed, it is likely that regulatory molecules and localization phenomena within marrow stroma are required to sustain and regulate hematopoietic function.[18] Stromal cells of the hematopoietic microenvironment provide the physical framework within which hematopoiesis occurs. They play a role in directing the processes by synthesizing, sequestering or presenting growth-stimulatory and growth-inhibitory factors, and also express a broad repertoire of adhesion molecules which mediate specific interactions with hematopoietic stem/progenitor cells.[19] Differential expression of adhesion molecules could cause different stem cell subsets to home to different marrow microenvironments capable of differently affecting self-renewal.

While much progress has been made in identifying cytokines and stromal factors, little is known about intracellular mechanisms regulating hematopoietic stem/progenitor cells self-renewal and differentiation.[20] Structure-function analysis of growth factor receptors as well as identification of novel signal transduction molecules have provided new insights into the processes involved in signal transmission pathways. Post-translational modifications of pre-existing proteins, in particular tyrosine phosphorylation, play a key role in transmitting signals and thereby linking extracellular signals to the activation of nuclear effector molecules which govern gene expression.[20] Accumulating evidence points to transcription factors such as AML-1, Ikaros, SCL/Tal-1, Rbtn-2, Tan-1, GATA-2, and HOX homeobox genes as important regulators of these processes. Overexpression of HOXB4 in murine bone marrow cells markedly increases the regenerative potential of long-term repopulating cells and causes an expansion in clonogenic progenitor cell numbers, without altering their ability to differentiate normally into mature myeloid, erythroid and lymphoid cells.[21] In contrast, overexpression of HOXB3 causes defective lymphoid differentiation and progressive myeloproliferation.[22] The glucocorticoid receptor, in combination with an activated receptor tyrosine kinase, seems to be a key regulator of erythroid self-renewal.[23] Shc overexpression increases GM-CSF sensitivity and prevents apoptosis of the GM-CSF-dependent acute myeloid leukemia cell line GF-D8, thus suggesting that Shc is an important regulator of cell survival and proliferation.[24] Different levels of protein kinase C modulate progenitor cell phenotype by favoring myelomonocytic or eosinophil differentiation.[25] Recently, it has been shown that telomerase expression correlates with hematopoietic self-renewal potential. Hematopoietic stem cells show decreasing telomere length with increasing age.[26] Thus, telomerase may regulate self-renewal capacity by reducing the rate of DNA shortening. Overall, intracellular mechanisms of hematopoietic control result in the repression or de-repression of lineage-specific genes regulating growth factor responsiveness and/or proliferation potential.[27] The exact knowledge of these mechanisms will greatly modify our approach to stem/progenitor cell manipulation.

In summary, stem and progenitor cell behavior is the result of highly integrated phenomena based on extracellular signals triggering intracellular transduction phenomena. The properties of self-renewal and differentiation give stem cells their remarkable ability to repopulate the hematopoietic tissue of lethally irradiated or genetically defective recipients. Understanding the interplay between extracellular and intracellular regulatory factors in controlling lineage determination remains an important challenge for the future clinical use of hematopoietic cells.

Stem cell antigen(s)

CD34 is a surface glycophosphoprotein expressed on early lympho-hematopoietic stem and progenitor cells, small-vessel endothelial cells, as well as embryonic fibroblasts.[28] $CD34^+$ hematopoietic cells are morphologically and immunologically heterogeneous and functionally characterized by the *in vitro* capability to generate clonal aggregates derived from early and late progenitors and the *in vivo* capacity to recon-

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stitute the myelo-lymphopoietic system in a myeloablated host.[29] The CD34+ cell population contains virtually all the myeloid and lymphoid progenitors as well as a small subset of cells that can initiate and maintain stromal cell-supported long-term cultures. Expression of the CD34 marker has dominated attempts to isolate, purify and characterize human hematopoietic stem cells by a variety of immunologic means.

Several monoclonal antibodies (MoAbs) assigned to the CD34 cluster identify a transmembrane glycoprotein antigen of 105-120 kd expressed on 0.5-2% normal BM cells, 0.01-0.1% peripheral blood cells and 0.1-0.4% cord blood cells.[30] The function of the CD34 antigen is not yet known, although it seems that CD34 is involved in stem/progenitor cell localization/adhesion in the marrow.[31] CD34 antigen expression is associated with the concomitant expression of several markers, including the lineage nonspecific markers Thy1, CD38, HLA-DR, CD45RA, CD71 as well as T-lymphoid, B-lymphoid, myeloid and megakaryocytic differentiation markers.[30] Analysis of the expression of CD38, Thy-1, CD71, the isoforms of CD45, and uptake of rhodamine-123 have resulted in a consensus stem cell phenotype which is $CD34^{bright}$, $Thy\text{-}1^{+}$, $CD38^{-}$, $CD45RA^{-}$, $rh\text{-}123^{dull}$, Hoechst 33342^{dull}, Lin^{-}. $CD34^{+}$ cells also express receptors for a number of growth factors classified as tyrosine kinase receptors, such as the stem cell factor receptor (SCF-R) or the stem cell tyrosine kinase receptors (STK), and hematopoietic receptors, not containing a tyrosine kinase domain.[32] Tyrosine kinase receptors are of particular relevance since their ligands might represent new factors able to selectively control stem cell self-renewal, proliferation and differentiation.[33] Recently, $CD34^{-}$ cells have been shown to have functional characteristics associated with stem cells and differentiate, *in vivo*, to $CD34^{+}$ cells.[34]

Stem/progenitor assays to evaluate engraftment potential

Different types of progenitors can be measured directly by multiparameter phenotyping of $CD34^{+}$ cells or subpopulations. Although this approach has the significant advantage that the results may be quickly available and can be used to guide clinical decisions, correlations between progenitor cell phenotype and functional activity are not yet refined enough to be clinically applicable. In addition, although CD34 antigen is expressed by virtually all progenitor cells, the percentage of $CD34^{+}$ cells with clonogenic activity *in vitro* ranges from 10 to 50%. Non-clonogenic $CD34^{+}$ cells include lymphoid progenitors as well as subsets of cells which are unresponsive to conventional growth factors and might require the presence of still unknown factors able to activate stem cell specific genes.[14] Figure 2 shows a schema of the cellular organization of hematopoiesis based on *in vitro* and *in vivo* functional properties of progenitor cells.

Recently, a new human hematopoietic cell, termed the SCID repopulating cell (SRC), that is capable of extensive proliferation and multilineage repopulation of the bone marrow of nonobese diabetic (NOD)/SCID mice has been identified.[35,36] The SRCs which are detected exclusively in the $CD34^{+}$ $CD38^{-}$ cell fraction have been shown to be biologically distinct from CFC and most LTC-IC.[35,36] With the exception of transplantation of human cells into immunodeficient mice, the identification of putative human stem cells has essentially relied on *in vitro* assays. Short-term *in vitro* assay systems require appropriate nutrients and growth factors and are particularly suitable for measuring quantitative changes of the different progenitor cell types as well as for evaluating growth factor responsiveness or investigating differential effects of regulatory molecules on progenitors at different stages of differentiation or on different hematopoiet-

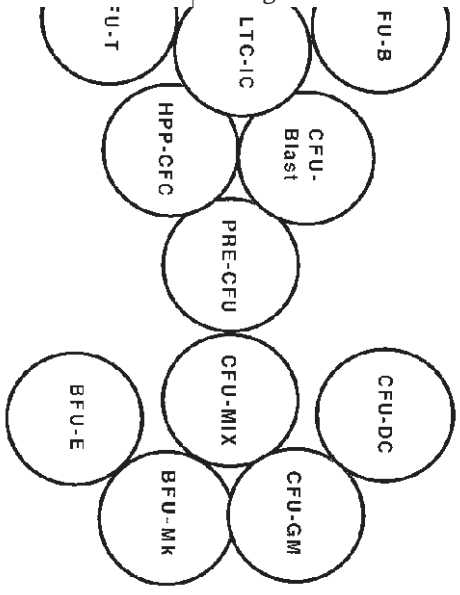

Figure 2. Cellular organization of hematopoiesis.

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ic pathways.[37] Short-term assays are not suitable for analyzing self-renewal or interactions of hematopoietic progenitors with stromal cells.

By using the long-term culture (LTC) technique, a sustained production of myeloid cells can be readily achieved *in vitro*, provided that a stromal layer is present, when marrow (or blood) is placed in liquid culture at relatively high cell concentration, with appropriate supplements, temperature and feeding conditions.[37]

The LTC system, based on the re-establishment *in vitro* of the essential cell types and mechanism responsible for the localized and sustained production of hematopoietic cells in the marrow *in vivo*, offers an approach able to investigate not only the proliferative and differentiative events but also self-renewal of any clonogenic cell types. A 5- to 8-week time period between initiating cultures and assessing clonogenic progenitor numbers allows a very primitive, self-renewing human cell, the so-called *long-term culture-initiating cell* (LTC-IC) to be quantified.[38,39] Limiting dilution assays allow the frequency of LTC-IC and their proliferative potential (number of CFU-GM generated by each LTC-IC) to be calculated. Another assay system, the cobblestone area-forming cell (CAFC), uses a pre-formed stroma as a support for hematopoiesis.[40] In this system, the primitive cells are measured directly by their ability to form characteristic colonies of cells resembling cobblestones.

With the possibility of studying not only the differentiation but also the self-renewal of primitive progenitors, the LTC system will play an increasingly important role in the design and assessment of new strategies involving the genetic engineering of hematopoietic cells and marrow stromal cells. Hematopoietic cells that can generate active hematopoiesis for weeks *in vitro* or months *in vivo* after transplantation are considered stem cells. This seems a clinically useful criteria, because it characterizes those cells which are important for sustained hematopoietic recovery following SCT. However, it reflects an oversimplification of the rather complex process of hematopoietic function. In fact, the ability of a cell to provide long-term hematopoietic activity can either be due to a long period of quiescence after the initiation of the culture or be a function of the probability of stem cell self-renewal which influences the long-term survival of stem cell clones.[41] Thus, the number of primitive cells measured in an LTC assay will be the product of the number of stem cells present at the onset of the culture and the probability of stem cell self-renewal. Although LTC assays will likely predict the *in vivo* repopulating activity of the graft, the clinical definition of a stem cell does not consider those stem cells that differentiate and die soon after transplantation or initiation of a culture.

Preparation of hematopoietic cells for *ex vivo* expansion

In the vast majority of cell culture systems, the presence of inhibitory mature and accessory cells limits the degree of *ex vivo* expansion of the progenitor cell compartment. Thus, a higher production of total cells, clonogenic cells and more immature hematopoietic progenitors has been observed when purified progenitor cells (namely $CD34^+$ cells) rather than the whole BM, cord blood.[42] or peripheral blood stem cell (PBSC) collections[43] are cultured *ex vivo*. Haylock *et al*.[43] selected and cultured 1,000 $CD34^+$ cells in presence of a 10-fold excess of contaminating $CD34^-$, $CD3^-$, $CD14^-$ cells and found no differences in total cell production after 14 days of culture, as compared to the production of 1,000 $CD34^+$ cells grown alone. However, when $CD34^+$ cells were mixed with increasing concentrations of $CD3^+$ $CD14^+$ cells, a marked decrease in the total cell output was observed after two weeks of culture suggesting an inhibitory activity of monocytes and T-cells. Despite the lack of information on CFU-C production, these results point out that the purity of the starting population is an important variable and it was recommended that at least 50% of cells should be $CD34^+$ in the initial cellular input.[43] There are also practical advantages in using a purified stem cell population as starting material for *ex vivo* manipulation, such as the ease of cell handling and the amount of cytokines consumed in the culture. Other variables that play an important role in stem cell expansion, which will be discussed in detail in the following paragraphs are: initial cell density, different cytokines used and their concentration, the presence of stromal cells, the composition of the culture medium and the refeeding schedule. Conversely, it has been recently demonstrated[44] that $CD34^+$ cells can be safely and efficiently processed after cryopreservation suggesting that the availability of fresh hematopoietic cells may not be an essential prerequisite for *ex vivo* expansion. Moreover, whereas the majority of preclinical and clinical studies[45] have attempted to optimize the expansion of highly enriched $CD34^+$ cells, under certain circumstances it may be advisable to select earlier subfractions of progenitor cells such as $CD34^+$ DR^- cells in chronic myelogenous leukemia (CML) or $CD34^+$ $Thy\text{-}1^+$ lin^- cells in multiple myeloma (MM). In these diseases the $CD34^+$ cell population is still contaminated, to various degrees, by malignant cells;[46-49] therefore, isolation of primitive progenitors prior *ex vivo* expansion may provide a starting cell population with a high proliferative potential free of tumor cells.

Methods for hematopoietic progenitor cell (HPC) enrichment

Several methods have been proposed for purification of HPC (Table 1). Their final target is a cell population with optimal purity, viability and high proliferative potential obtained by means of a low cost, rapid and simple separation technique. Early attempts toward the purification of HPC were based on the cell physical properties. Density-gradient centrifugation, velocity sedimentation and elutriation are methods

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Table 1. Hematopoietic cell separation systems.

	Recovery (% of initial cells)	Purity (% CD34+ cells)	Clinical grade device
Immunomagnetic beads			
Negative selection	20-50	20-60	Yes
Positive selection	30-60	50-90	Yes
MACS	>80	>90	Under evaluation
Panning	30-50	40-70	No
Avidin-biotin immunoabsorption	40-60	50-90	Yes
FACS (high-speed cell sorting)	30-50	>95	Yes

that separate cells based on cell size and buoyant density. More recently, immunologic selection techniques which take advantage of the expression of specific antigens on HPC membrane, have allowed a much better degree of enrichment. Specifically, the demonstration of the presence of the CD34 antigen on HPC[28] has led to a number of positive selection systems which use MoAbs to purify hematopoietic precursors.

Alternatively, $CD34^+$ cells can be enriched by depletion of $CD34^-$ accessory and mature cells.

Fluorescence activated cell sorter (FACS)

Flow cytometry can be used to separate HPC from a heterogeneous population after incubation of cells with fluorochrome-conjugated MoAbs directed to cell-surface markers. Moreover, multiparameter enrichment can be obtained by combining physical properties such as cell size and cytoplasmic granularity and intracellular characteristics indicating cellular function (e.g. propidium iodide to determine cell viability; rhodamine-123 to assess metabolic quiescence and nucleic acid dyes to evaluate cycling status). This cell sorting technique can yield a highly purified (> 99%) cell population combining positive and negative markers for HPC using clinically-graded MoAbs. The main criticisms to the use of flow cytometry for selecting large numbers of cells are the low recovery of target cells and the length of time required to process the whole BM harvest or the leukapheresis products. The development of multiparameter high-speed cell sorting has been described and recently upgraded for clinical use.[50,51] Viable cells have been sorted at rates as high as 40,000 cells/sec as compared to 2,000-5,000 cells/sec of commercially available cell sorters. Thus, the sample processing time can now be reduced to 8-12 hours. The sorted cell fraction also maintains its hematopoietic potential based on the presence of CFU-C, more immature CAFC and long-term repopulating cells in mice.[51] Presently, selection of HPC (i.e. $CD34^+Thy\text{-}1^+lin^-$ cells) from clinical samples is directed toward the purification of cell populations highly enriched for HPC free of contaminating malignant cells in myeloma patients.[47]

Panning

Anti-CD34 MoAbs bound to the bottom of cell culture flasks have been used to select $CD34^+$ cells.[52] The target cell population present in a heterogeneous cell suspension is blocked on the plastic surface while $CD34^-$ cells remain in the supernatant and can be easily eliminated.

Despite the good results reported, the availability of more efficient methods of cell separation have made this technique largely redundant.

Immunomagnetic systems

A variety of magnetic cell-separation methods have been described.[53] Some of these systems are commercially available and have been used in clinical trials. The main differences between the currently used magnetic cell-separation methods are the composition and size of the magnetic particles used for labeling the cells and the separation process. Superparamagnetic beads can be equally used for negative and positive cell separation depending on the specificity of MoAbs. The rosetted target cells can be easily isolated from unlabeled cells by a magnet applied on the outer wall of the test tube or blood bag. Large magnetic beads (diameter >0.5 μm) have been used clinically for the purging of neuroblastoma and lymphoma cells from stem cell harvests prior to autologous transplantation.[53,54] Immunomagnetic beads coupled with anti-CD34 MoAb can be used for positive selection of HPC.[55] However, before the clinical use of the enriched cell fraction, the cell-bound particles must be removed to avoid damage to the cells and/or toxic events to the patient. Beads can be released using chymopapain or a peptide competing with the CD34 Ab. In alternative, HPC can be enriched by negative depletion of mature and accessory cells targeting lineage-specific antigens.

More recently, the magnetic cell-sorting (MACS) system has been proposed as an efficient and more manageable alternative to flow cytometry for cell separation.[56] It uses colloidal-sized superparamagnetic particles made of dextran and iron oxide with 60 nm of diameter. The use of very small beads minimizes unspecific binding and allows the efficient isolation of rare cells. In addition, the magnetic particles are readily internalized by the labeled cells without affecting their physical, phenotypic and functional capacity.[57] Table 2 reports the results of a large number of experiments (=14) comparing the efficiency of the Mini-MACS system for selecting $CD34^+$ cells from two different cellular sources (R.M.L., *unpublished observations*).

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High-affinity chromatography based on avidin-biotin immunoabsorption

This technique relies on the high affinity between the protein avidin and the vitamin biotin whose interaction has an extremely high dissociation constant (= 10^{-15} M). In this system, a heterogeneous cell population is incubated with a biotinylated antibody to the CD34 antigen. The cell mixture is then passed through a disposable column containing avidin-coated polyacrylamide beads. $CD34^+$ cells are retained on the column due to the high affinity binding of biotin to avidin while negative cells are washed away. Target cells are then recovered by mechanical agitation of the column which disrupts the antibody-antigen link. Thus, bound cells are eluted from the column mainly free of antibody. An automated version of the device controlled by a computer which guarantees reproducibility and reduces risks of operator errors has been developed and used in the setting of autologous and allogeneic stem cell transplantation.[46, 58]

Dynamic systems

In addition to positive or negative selection of $CD34^+$ cells, effective *ex vivo* expansion of hematopoietic progenitor cells and, perhaps, putative stem cells can also be achieved in the presence of unselected cell populations. In this case the use of *dynamic* perfusion cultures is strictly required. As stated above, the production of inhibitory factor(s) by mature and accessory cells rather than the availability of growth promoting factors, is probably the main limitation to successful stem cell expansion. For instance, it is well known that suboptimal cell expansion occurs when $CD34^+$ cells are cultured at concentrations exceeding 10^4 cells/mL. Moreover, the presence of stromal feeder-layer cells seems to be important for effective BM stem cell expansion induced by exogenous cytokines. Therefore, an artificial capillary-perfusion system (Bioreactor) in which nutrients consumed by proliferating cells are continuously replenished by exchange of the nutrient-depleted medium with fresh culture medium, has been tested for *ex vivo* expansion of hematopoietic cells co-cultivated with stroma cells or stromal cell lines.[59] Cytokines such as IL-3, IL-6 and GM-CSF have been added to the culture to optimize cell expansion and to provide growth factors which are not produced by stromal cells (i.e. IL-3). In this system, cultured cells are confined in a small compartment separated from a large medium reservoire. Medium exchange is optimized when there is a maximal membrane surface area per unit volume across which medium and nutrients can pass by diffusion. This is best achieved by a capillary-perfusion module. Three important requirements for optimal expansion of hematopoietic cells are: the capillary porous size, the capacity of supporting the attachment of stromal cells by the module and the minimal cell activation by the materials of the module. In fact, mature myeloid cells such as macrophages release ,upon surface activation, tumor necrosis factor(s) (TNFs), interferon(s) (IFNs) and other substances which negatively affect stem cell expansion.

Table 2. High efficiency of Mini-MACS separation system for the enrichment of BM or circulating $CD34^+$ cells.

Source	Enrichment Pre (%)	Enrichment Post (%)	Recovery (%)	CE*	LTC-IC ($x10^4$ $CD34^+$)
BM	2.3±1	97±3	88±9	3.6±0.3	62.5±54
PB	0.7±0.4	98.9±1	90±8	3.9±0.4	48.2±35

**Abbreviations: CE, clonogenic efficiency; BM, bone marrow; PB, peripheral blood. The results are expressed as mean±SD.*

Bioreactors have induced a remarkable expansion of committed hematopoietic progenitor cells coupled with a modest increase in the number of LTC-IC,[59] a population of primitive cells which correlates most positively with the long-term reconstituting capacity of autologous and allogeneic grafts.

Ex vivo expansion of myeloid progenitor cells

Ex vivo expansion of hemopoietic progenitors might result in:

- amplification of the population of committed progenitors due to an extensive, although controlled, differentiation process;
- amplification of the stem cell pool through extensive self-renewal of the early progenitor cell population.

Obviously, both processes can take place simultaneously mimicking, *in vitro*, the complex interplay of regulatory mechanisms that allows hemopoiesis *in vivo*. The latter situation, so far, has never been obtained *in vitro*, whereas different approaches have permitted the first goal to be reached (at least, to a certain extent) and some recent data suggest that relevant self-maintaining processes can be triggered.

The clinical relevance of extended differentiation versus self-renewal is obviously different. The induction of an increased *in vitro* production of committed progenitors might hasten the early phases of hemopoietic reconstitution which occur after myeloablative treatment and stem cell transplantation. Moreover an increased number of infused cells might modulate graft versus-host disease (GVHD) intensity in the allogeneic setting.[60]

Although relevant, the potential clinical benefit of techniques allowing only committed progenitor cell expansion is outweighed by the possibility of triggering the self maintenance, and perhaps amplification, of early hemopoietic progenitors. In this situation, starting from a limited number of progenitors, long-

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term reconstitution of hematopoiesis might become feasible. Moreover, *ex vivo* manipulation of primitive hemopoietic cells could be performed under experimental conditions suboptimal for the growth of neoplastic cell contaminating autologous grafts. Thus, a purging effect could be obtained.

Several attempts of *in vitro* expansion of hemopoietic progenitors have been published in the last years. Recent reviews[61, 62] summarize early experiences.

The first studies on *ex vivo* generation of hematopoietic progenitors involved liquid culture in the presence of cytokines such as SCF, IL-1, IL-3, IL-6, G-CSF, GM-CSF and Epo. These experiences showed that a relevant increase (from 10 to 1,000 fold) of $CD34^+$ cells and of committed progenitors can be obtained. The expansion of committed progenitors does not mean, however, that the long-term reconstitution of hemopoiesis is achievable. Indeed, several data support the concept that an uncontrolled commitment decreases the stem cell pool. Yonemura *et al.*[63] have reported that IL-3 or IL-1 abrogates the reconstituting ability of hematopoietic stem cells. Furthermore, Peters *et al.*[64] have demonstrated that *ex vivo* expansion of murine marrow cells with IL-3, IL-6, IL-11 and SCF leads to impaired engraftment in irradiated hosts.

As indicated by Traycoff *et al.*[65] *ex vivo* expansion of hematopoietic cells using SCF, IL-1α, IL-3 and IL-6 generates classes of cells possessing different levels of BM repopulating potential based on their cycling status. Along this line, Young *et al.*[66] correlated a higher proliferative capacity with a quiescent status after *ex vivo* expansion in the presence of SCF, IL-3, IL-6 and LIF. Taken together, these data suggest that cultures in the presence of cytokine combinations based upon SCF, IL-1 and IL-3 involve differentiation of BM or mobilized $CD34^+$ cells entering the S phase. Different results were obtained by Di Giusto *et al.*,[67] who found that *ex vivo* expanded cord blood $CD34^+$ cells repopulated the marrow of immunodeficient mice as well as non-expanded cells. However, it must be remembered that cord blood is rich in hemopoietic progenitors[68] that have an increased proliferation potential.[69]

New strategies to induce the expansion of $CD34^+$ cells with little (or no) differentiation might involve different approaches. The use of stirred suspension[70] or hollow fiber[71] bioreactors has been proposed in order to grow cells in a more physiologic environment, and inhibitors such as TGF-β and MIP-1α have been the object of intense studies. Recently, MIP-1α has been found to exert a weak inhibitory effect on $CD34^+CD38^-$ cells and to enhance the proliferation of $CD34^+CD38^+$ cells, whereas TGF-β strongly inhibits both cell populations.[72,73] The most promising results, however, have been obtained with the recent introduction of FL and Tpo. FL, a recently discovered member of the class III tyrosine kinase receptor family,[74] is able to induce proliferation of very early hematopoietic progenitors that are nonresponsive to other early acting cytokines, and to improve the maintainance of progenitors *in vitro*.[75-77] This is also supported by the finding that FL significantly reduced the number of cultured cells undergoing apoptosis.[78] Analysis of the effects of 16 cytokines on $CD34^+$ $CD38^-$ cells showed that FL, SCF and IL-3 produced a 30-fold amplification of the input of LTC-IC.[79] Yonemura *et al.*[80] compared FL- and SCF-driven *ex vivo* expansion. They reported that both cytokines, in combination with IL-11, enhanced the production of progenitors, but with different kinetics. In fact, the maximal expansion by FL required a longer incubation than with SCF. Interestingly, in these studies the combination of SCF/IL-11, together with IL-3, reduced the ability of cultured cells to reconstitute hematopoiesis in irradiated hosts.[80] Other recent data have compared the effect of FL and SCF. FL acts as a self-renewal or proliferation/expansion signal for $CD34^+$-low cells while the effect of SCF is more likely to transduce a differentiation signal, resulting in more rapid repopulation at the expense of cell expansion.[81] Gene transfer studies in mice have also demonstrated that FL maintains the ability of human $CD34^+$ cells to sustain long-term hematopoiesis. In fact, incubation of $CD34^+$ cells with FL before transduction was associated with long-term provirus expression, whereas provirus expression declined in recipients of $CD34^+$ cells transduced in the absence of FL.[82] The expansion *ex vivo* of early progenitors seems to be affected at the single cell level by changes in cytokine concentrations. In a recent paper by the Vancouver group,[83] maximal LTC-IC expansion was obtained in the presence of 30 times more FL, SCF, IL-3, IL-6 and G-CSF than could concomitantly stimulate the near-maximal amplification of CFC.

Tpo, the ligand of the mpl receptor expressed on both early and committed hematopoietic progenitors,[84] is known to support megakaryocytopoiesis.[85] Moreover, it has been shown to be capable of enhancing *ex vivo* expansion of early/committed progenitor cells. As single factors, FL and Tpo stimulated a net increase of LTC-IC generated from $CD34^+$ $CD38^-$ cells within 10 days.[79] Furthermore, as demonstrated in mice recipients of BM cells transduced with the mpl receptor,[86] Tpo does not induce lineage-restricted commitment of mpl-receptor positive pluripotent progenitors but permits their complete erythroid and megakaryocytic differentiation. Tpo has also been found to increase the multilineage growth of $CD34^+$ $CD38^-$ cells from 3%, in absence of the cytokine, up to 40% when Tpo is added to SCF and FL.[87] The presence of additional cytokines such as IL-3, IL-6 and Epo does not significantly enhance clonal growth above that observed in response to Tpo, SCF and FL.[87] Interestingly, the soluble form of Tpo receptor and G-CSF receptor directly stimulate the proliferation of primitive hematopoietic progenitors of mice in synergy with SCF and FL.[88]

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A step toward extensive *ex vivo* amplification of early human progenitor cells has been reported by Piacibello *et al*.[89] They first demonstrated that IL-3 induces an early production of committed progenitors but is not able to sustain true self maintenance of hemopoietic stem cells even in the presence of other early acting cytokines (FL, Tpo, SCF). Afterwards, several combinations of early acting cytokines were tested for their ability to sustain long-term hematopoiesis in stroma free cultures. Among the various combinations tested on purified cord blood CD34$^+$ cells, the mixture of Tpo + FL was found to be able to maintain early progenitors up to six months.[89] These data indicate the enormous potential of cord blood progenitors and the key role of Tpo and FL in the regulation of early hematopoiesis. However, several issues remain to be clarified:

- is it true self-renewal or a slow differentiation of cord blood cells, which are rich in immature progenitors?
- what is the *in vivo* repopulating capacity of *ex vivo* expanded cells?
- is such expansion possible using CD34$^+$ cells obtained from the marrow or peripheral blood of adult subjects?

In this view, while a number of papers have already reported that committed progenitors can be generated and safely administered to transplant recipients, there are no reports on expansion of cells with long-term repopulating capacity in humans.

Brugger *et al*.[45] reported the successful reconstitution of hematopoiesis in ten cancer patients transplanted with autologous cells generated from CD34+ cells cultured in the presence of SCF, IL-1β, IL-3, IL-6 and Epo. However, the conditioning regimen given to these patients was not fully myeloablative, and this study offered no insight into the long-term engraftment potential of cells generated in this fashion. A similar approach was followed by Alcorn *et al*.[44] In ten patients with malignancy, an aliquot of the PBSC harvest was recovered from liquid nitrogen and CD34 were selected. Cells were cultured for 8 days in the presence of the same cytokine combination. A mean of 379×10^6 expanded cells were reinfused in addition to unmanipulated cells. The authors reported that the total LTC-IC number was not increased and most of the CD34$^+$ cells were differentiated in front of an average 15-fold CFU-GM expansion (range 4-39). Similarly, averages of 71 (range 27-151)-fold megakaryocytic cell expansion and 1,040 (19-16.000)-fold erythroid cell expansion were reported. Although adverse reactions were not reported, no difference in the kinetics of engraftment was observed in comparison with historical controls.

Different results come from preclinical studies where the infusion of large numbers of *ex vivo* expanded committed hematopoietic progenitors, together with unmanipulated cells might speed engraftment after chemotherapy and/or total body irradiation (TBI). Data from Uchida *et al*.[90] have suggested in the past that most of the short- as well as the long-term engraftment potential resides in uncommitted progenitors. More recently, Szilvassy *et al*.[91] have demonstrated that partially differentiated *ex vivo* expanded cells accelerate hematologic recovery in myeloablated mice transplanted with highly enriched long-term repopulating stem cells. In humans, Williams *et al*.[92] reinfused 9 breast cancer patients with unmanipulated apheresis products together with a mean of 44×10^6/kg mature CD15$^+$ cells generated *ex vivo* by CD34$^+$ cells cultured in the presence of PIXY-321. No toxicity was observed after reinfusion, and time of white cell recovery was similar to that observed in the retrospective control group. In a more recent study,[78] megakaryocytic progenitors (MP) were obtained from CD34$^+$ cells cultured in serum-free medium in the presence of Tpo, FL, SCF, IL-3, -6, -11 and MIP-1α. Proliferation peaked on day 7 in culture, and a 8±5-fold expansion of CD34$^+$/CD61$^+$ cells, a 17±5-fold expansion of CFU-MK and a 58±14-fold expansion of the total number of CD61$^+$ cells was obtained. Ten cancer patients undergoing autologous PBPC transplant received MP generated *ex vivo* (range 1-21 CD61$^+$ cells $\times10^5$/kg) together with unmanipulated PBSC. Platelet transfusion support was not needed in 2 out of the 4 patients receiving the highest dose of cultured MP and this result compared favorably with a retrospective control group of 14 patients, all requiring platelet transfusion support. A major concern is the potential expansion of contaminating tumor cells along with hematopoietic progenitors. In fact, it has been demonstrated that CD34$^+$ cell selection decreases (but does not abrogate) neoplastic cell contamination from aphereses of myeloma patients.[46] For instance, in the majority of B cell lymphoma patients CD34$^+$ cell selection does not eliminate contaminating t(14;18)$^+$ cells. However, during *ex vivo* expansion residual lymphoma cells do not proliferate and become undetectable by molecular analysis in the majority of cases.[93] Similarly, Vogel *et al*. recently indicated that exogenously mixed epithelial tumor cell lines might have a relative disadvantage over CD34$^+$ cells during *ex vivo* expansion.[94]

Future directions

Future challenges in this field are represented by the expansion of true human *stem cells* without maturation, to extend this strategy to allogeneic stem cell transplantation, and especially cord blood allograft, as well as the manipulation of cycling of primitive progenitors for gene therapy programs.

Selective amplification of specific myeloid lineages (e.g. platelets or granulocytes) may improve the results of autologous transplantation. Moreover, although early results need confirmation, the amplification of early/committed hematopoietic cells coupled with the removal of neoplastic cells contaminating autologous grafts appears to be feasible.

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Expansion of cytotoxic effectors

Human cytotoxic effector (CE) cells can be divided in two major groups:

1. cells requiring prior antigen sensitization, which recognize their target in the context of the major histocompatibility complex (MHC) molecules;
2. cells not requiring prior antigen sensitization being spontaneously cytotoxic against tumor target cells (e.g. K-562 cell line) in a non-MHC restricted setting.

While the first group includes only some subsets of T-lymphocytes ($CD8^+$ or $CD4^+$ cells), the second one is more heterogeneous and includes both T-cells and natural killer (NK) cells, expressing the CD56 antigen.[95]

The so-called antibody-dependent cellular cytotoxicity (ADCC) can be mediated by cells expressing the Fcγ receptor II and the Fcγ receptor III (e.g. NK cells and $CD3^+/CD16^+$ cells). Although this activity is not exhibited by non MHC-restricted cells, it cannot be considered aspecific and it is also exerted by monocytes.

The lymphokine-activated killer cells (LAK) are capable of killing NK-resistant cellular targets (e.g. Daudi cell line). Although some tissue-resident lymphocytes may have spontaneous LAK activity, normal blood mononuclear cells do not show any LAK activity, which can be acquired after incubation with Interleukin-2 (IL-2).[96]

Therefore the LAK assay is a measure of the capacity of T and NK cells to become activated and to express cytolytic function.

Killing mechanisms of cytolytic effectors

Cytotoxic T-Lymphocytes (CTL) and NK cells possess at least two distinct, fast-acting, lytic mechanisms:[97, 98]

1. the granule exocytosis pathway involves the secretion of perforin and granzymes which penetrate throughout the target cell pores, inducing cell death;
2. a non-secretory mechanism which is mediated by the interaction between the Fas-ligand, expressed by the killer-cell and Fas (CD95) which triggers a cascade of proteolytic enzymes leading to apoptosis of both the killer and the target cell.

A third cytolytic pathway, involving TNF, has recently been described.[99]

A series of apoptosis-resistant clones of human lymphoma cells has been described. These cells express fas/APO-1 receptors lacking the intracytoplasmic signaling domain.[100]

NK cells, as well as CTL, can recognize MHC class I molecules. However, recognition of MHC on target cells downregulates the NK cell function, suggesting the presence of inhibitory receptors.[101]

LAK cells are not MHC-restricted and are also capable of killing freshly isolated tumor cells. Similarly to CTL and NK cells, their activity is mediated by both lytic pathways (perforin/granzyme) and Fas-mediated apoptosis.

Cytokines involved in CE function

Several cytokines affect CTL and NK cell response. In particular, IL-2 expands the pool of alloreactive CTL precursors and IL-15 (produced by monocytes) mimics IL-2 action by inducing γ-IFN production, the activation of memory T cells and CTL proliferation.[102]

In addition, GM-CSF can affect certain T-lymphocyte functions by enhancing their cytotoxicity and γ-IFN production. This multifunctional cytokine can also augment NK cell function and the expression of adhesion molecules on the surface of leukemic cells.[103,104] Moreover, it has been hypothesized that the association of GM-CSF/IL-2 can also be useful for the activation of cytotoxic effectors by circulating progenitors, preserving the clonogenic potential of normal hemopoietic precursors.[105]

IL-12 elicits the production of γ-IFN by CTL thus enhancing their antineoplastic efficacy and promotes the differentiation of T-helper-1.[106,107] Finally, IL-7 seems to be critical for the development of CTL and for a fast immune reconstitution after bone marrow transplantation (BMT).[108]

In conclusion, these cytokines play a pivotal role in the immune response against tumor cells by expanding, activating and recruiting CE and secondary effector cells (macrophages) or by directly inhibiting tumor cell growth.

Role of cytotoxic effectors in immunosurveillance

There are several *in vitro* and *in vivo* data supporting the role of immunosurveillance in tumor growth control.[109] The graft-versus-leukemia (GVL) effect has been demonstrated to play a critical role in the eradication of minimal residual disease (MRD) after allogeneic trasplantation and there is evidence supporting the role of both T cells and NK cells in preventing disease relapse.[110]

Today, there is no doubt that CTL have a major role in killing allogeneic tumor cells in a MHC-restricted manner. For example, in CML, the higher relapse incidence after T-cell depleted allogeneic BMT[111] and the dramatic effect of donor T-lymphocyte infusion after relapse following BMT,[112] strongly support the importance of MHC-restricted GVL.

Unfortunately, a selective GVL effect (separated from GVHD) can be obtained very rarely in patients receiving allogeneic BMT. Moreover, although animal models indicate that autologous GVHD exists and could generate a significant antitumor effect, the high incidence of relapse in patients receiving autologous BMT demonstrates that autologous GVL is often clinically ineffective.[113]

Rationale for cytotoxic effectors' expansion

There are many important reasons to increase the number, the efficacy and the specificity of cytolytic

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effectors both in the allogeneic and in the autologous setting. The main clinical goals are the following:

1. to reduce the relapse-incidence after autologous BMT, which is still relevant in acute leukemia, lymphoma and breast cancer;
2. to cure diseases in which autologous BMT can only prolong the survival (multiple myeloma, CML, metastatic chemosensitive cancers);
3. to reduce the incidence of relapse after allogeneic BMT especially in patients transplanted with great tumor burden;
4. to accelerate the immune reconstitution after BMT, in order to reduce the morbidity and transplant-related-mortality caused by serious infections (e.g. CMV and systemic mycosis).

The CE which are investigated for *in vivo* or *ex vivo* expansion are mainly NK cells in the autologous setting and CTL in the allogeneic setting.

Development and expansion of NK cells

NK cells belong to the naive part of the immune system and begin to appear into PB early after allo and auto-BMT. These cells express the N-CAM homologous CD56 antigen, but lack T-cell receptor α/β complexes. They are also characterized by low affinity receptors for IgG (CD16)[114-116] and their binding to malignant cells is mediated by CD18 molecule.[117] The most important organ for NK differentiation is the BM. Several data suggest that a common precursor of T cells and NK cells does exist. Fetal NK cells express the TCR γ, δ, ϵ, ξ sub-units while fetal B precursors do not express TCR subunits (Figure 3).

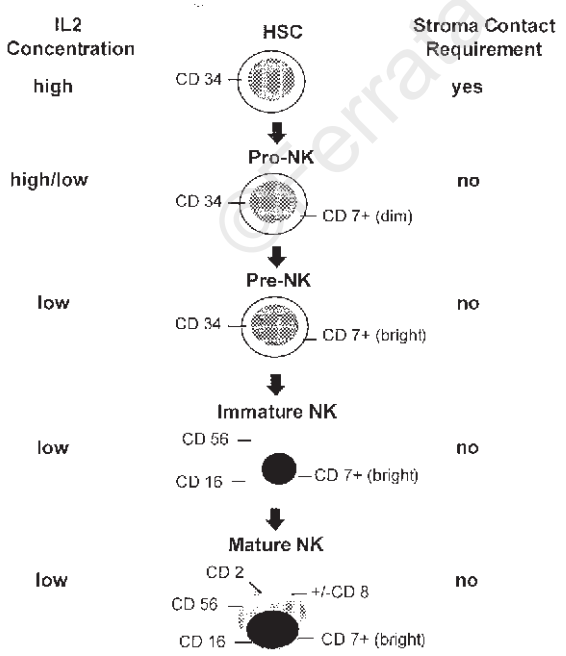

Figure 3. NK cells differentiation pathway.
Modified from Miller et al, Blood 1994; 83: 2594-601.

These bipotential T/NK precursors do not have TCR rearrangement and share CD34/CD33/CD7 antigens. They differentiate to NK in the presence of SCF, IL-7, IL-2 and stromal feeder cells. The first step of NK differentiation is stroma-dependent while the second step of NK maturation is stroma-independent and is strongly potentiated by the association of IL-2 with IL-7.[118] In all studies IL-2 is required for NK cell differentiation from $CD34^+$ cells. This finding suggests that a fraction of $CD34^+$ cells expresses IL-2 receptors and that activated T-cells (as IL-2 source) should be detectable in the cellular milieu. However, T-cell deficient mice have normal NK cell development and humans lacking the γ-chain subunit of IL-2R lack NK activity. These unexpected observations have recently been supported by the demonstration that IL-15, produced by BM stromal cells, can directly induce $CD34^+$ cells to differentiate into $CD3^-/56^+$ NK cells in the absence of IL-2.[119]

The last step of NK development, after the expression of CD16, is characterized by the appearance of the CD56 molecule. The intensity of CD56 expression directly correlates with the proliferative potential and the killing ability of NK cells.[120]

There is clear evidence that mature NK elements have a clonally-distributed ability to recognize their target cell by class I MHC alleles and a precise correlation has been established between the expression of p58 receptors on NK cell surface and class I MHC alleles. These receptors transduce an inhibitory signal upon interaction with MHC class I antigens, to prevent NK cells from killing target cells expressing certain (self) HLA alleles.

These findings are consistent with a self-tolerance mechanism exerted by the NK population which can be disrupted as a consequence of tumor transformation or viral infection or any other events inducing (or masking) class I molecules.[121,122]

After incubation with IL-2, NK cells become LAK cells capable of killing otherwise NK-resistant target cells. These (NK) activated cells express new markers such as CD25, MHC class II and fibronectin which can be useful for the evaluation of their functional state. The NK cell compartment is heterogeneous and distinct subsets have been characterized. The most informative functional differences are based on relative CD56 fluorescence: only $CD56^{+bright}$, but not $CD56^{+dim}$ NK cells, express the high-affinity IL-2 receptor. As a consequence, they respond to low concentrations of IL-2 and expand 10 times more than $CD56^{+dim}$. This subset seems to be significantly reduced in leukemic patients. A remarkable reduction of $CD56^{+bright}$ NK cells has been observed in CML patients coupled with a significant decrease of their spontaneous cytotoxicity against the K-562 line. However, this defect was corrected by 18 hours incu-

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bation with 1000 U/mL of recombinant IL-2.[120] These data strongly suggest that during tumor progression, the NK compartment (and particularly the small fraction of NK-CD56$^{+bright}$ with high proliferative ability) is progressively suppressed even though the exogenous administration of IL-2 can partially reverse this phenomenon. The strong correlation between functional capacity of the NK cell compartment and tumor progression has often been reported as well as the efficacy of the administration of LAK cells plus IL-2 in restoring an anti-tumor response.[123]

Along this line, more than 90% of patients with acute leukemia in complete remission do not show spontaneous cytotoxicity against autologous blast cells. However, *ex vivo* treatment with IL-2 restores cytolytic activity in 37.5% of these patients.[124]

The first attempts to generate and expand LAK activity either *in vivo* or *in vitro* (after *ex vivo* incubation with IL-2) have been clinically disappointing especially in patients autotransplanted for acute lymphoblastic leukemia (ALL).[125] Nevertheless, there is now a renewed interest in the use of activated NK cells in hematologic malignancies, based on the optimization of different approaches:

- administration of IL-2 *in vivo* to expand functionally active CE in patients with low tumor-burden, in order to reach an optimal effector/target ratio;
- harvesting and culturing large amount of NK cells for additional *ex vivo* expansion/activation with IL-2. Expanded cells should be reinfused in the early phase after BMT;
- sequential combination of both techniques (Figure 4).[126]

The systemic administration of IL-2 for *in vivo* expansion and activation of the NK compartment may theoretically have some advantages:

1. high number of CE precursors in the body;
2. possibility of activating CE residing in the tumor bulk;
3. more feasible and cheaper strategy than the generation and administration of LAK cells.

On the other hand, the main drawbacks of this approach are the high toxicity of systemic administration of IL-2 and the high variability of anti-tumor response.

Sources of cytotoxic effectors and modalities of NK cell expansion

Human NK progenitor cells can normally be found in the BM,[127,128] and originate from CD34$^+$ hematopoietic progenitors.[129] So far, the generation of NK cells from CD34$^+$ precursors has been described on a small scale basis but not in large scale experiments.[130]

High numbers of functionally active NK cells can be easily demonstrated in mobilized cells from patients receiving chemotherapy plus G-CSF. Silva *et al*.[131] have shown a 5.4-fold expansion of NK cells from leukapheresis products incubated in the presence of IL-2 for 6-8 days without affecting the CD34$^+$ cell content. However, decreased function of NK cells has recently been described in the PB of normal donors after G-CSF administration.[132] Circulating NK progenitors showed a decreased killing capacity and diminished proliferative ability in response to IL-2, as compared to their unprimed BM counterpart.

Based on previously published LAK trials,[123, 133] it can be estimated that about 10^{10}-10^{11} activated NK cells are needed to stimulate an anti-tumor response. A 100-fold *ex vivo* expansion of these cells from a standard leukapheresis collection would, therefore be required to obtain such a high number of effectors.

Beaujean *et al*.[134] reinfused autologous BM cells incubated for 10 days with IL-2 in 5 ALL patients following a myeloablative treatment. This procedure resulted in an important loss of hemopoietic progenitors with delayed engraftment. Moreover, in spite of this attempt to induce an autologous GVL, all patients eventually relapsed.[134]

Wong *et al*.[135] compared the ability of IL-2 alone or combined with IL-7 or IL-12 to stimulate NK activity in BM or PB samples. They found that IL-2/IL-12-activated blood cells suppressed the growth of the leukemia cell line K-562 about eight-fold more efficiently than BM cells. They also found that cryopreservation and subsequent stimulation of BM and PB cells did not significantly decrease the activity of NK cells. Finally, the combination of IL-2 and IL-12 showed a synergistic effect on both BM and PB elements.[135] Large-scale *ex vivo* expansion of NK cells for adoptive immunotherapy not only requires an optimal source of precursors, but also clinically approved materials and procedures. In this context, Miller *et al*.[136] obtained a 21-fold expansion of NK cells using a 21-day large scale NK culture performed in gas-permeable bags. Pierson *et al*.[137] observed a 352-fold expansion of NK cells after 33 days of incubation in a bioreactor. Their starting population was NK precursors enriched by negative panning with anti-CD5 and anti-CD8 antibodies. The activated NK population was highly purified (>90%) in CD56$^+$/CD3$^-$ cells and maintained a powerful cytotoxicity against K-562 cells.

The use of a homogeneous NK cell fraction for cell-therapy programs seems to be advantageous because activated NK cells have more specific lytic activity than heterogeneous LAK populations.[138] However, creating such a fraction requires a first step of enrichment (e.g. by eliminating CD8$^+$/CD5$^+$ cells) and long-term cultures carry the risk of fungal or bacterial contamination.

Cytokine induced killer cells (CIK) expansion

In 1986 Lanier described a subset of CD3$^+$ T cells co-expressing the CD56 antigen which is a typical NK marker.[139] A remarkable expansion of this cellular subset has recently been obtained by Schmidt *et al*.[140] following a 16-day incubation in the presence of IFNγ, IL-1, IL-2 and a monoclonal antibody against

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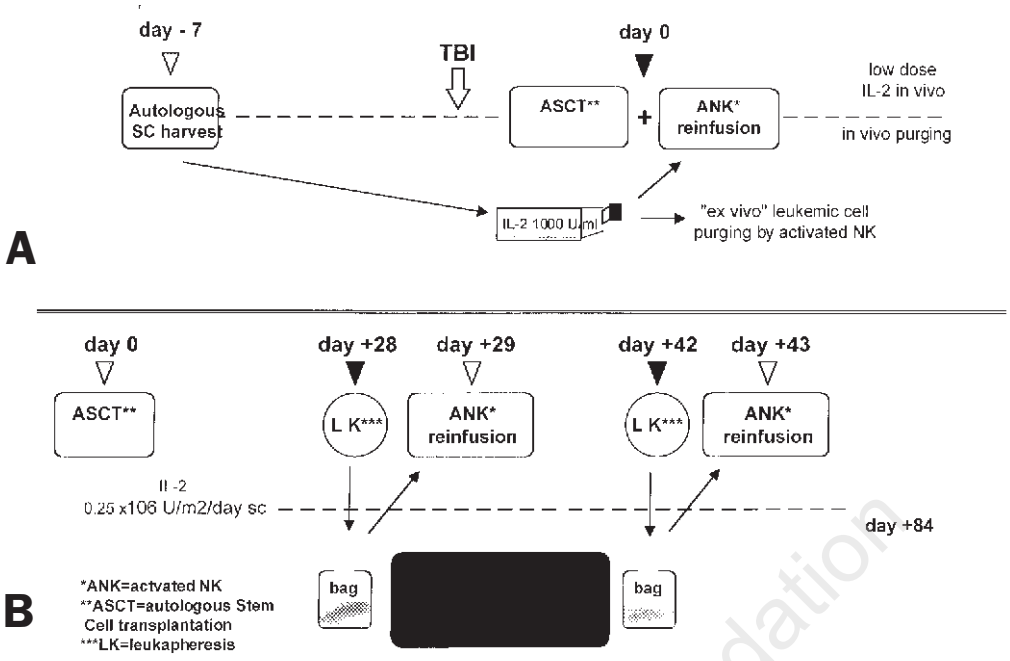

Figure 4. Strategies for generation and activation of NK cells *in vitro* and *in vivo*. Modified from Klingemann et al., Exp Hematol 1993; 21:1263-70.

CD3 as the mitogenic stimulus. The ability of CIK cells to deplete leukemic cells from CML marrow was then investigated by the same group.[141] While standard LAK cells were, in most cases, unable to lyse CML cells, CIK cells were toxic to both autologous and allogeneic CML blasts without affecting normal hemopoietic progenitors.

CTL expansion for adoptive therapy

It is well known that the GVL effect can be transferred with donor-buffy-coat (BC) lymphocytes.[142] The antitumor effect of the CTL contained in the BC has been shown to be more potent than that induced by NK cells, even though NK cells exert a GVL activity different from GVHD.[143]

In a murine model, a single dose of 2×10^7 CTLs in tumor bearing mice (DBA/2) resulted in the eradication of primary cancer and metastases without causing severe GVHD.[144] Unfortunately, this GVL effect cannot be easily separated from GVHD in humans. As a matter of fact, in clinical studies the beneficial effect of CTL administration is often offset by the severity of GVHD or marrow aplasia.

Although leukemic cells share common antigens with other tissues of the host, there is also the chance that distinct leukemic antigens may be recognized by specific allogeneic CTL.[145]

Leukemia-specific T-cell clones have been isolated from HLA-identical siblings[146] and this finding may explain the high incidence of CR, without GVHD, in patients with CML relapsed after allo-BMT and treated with donor BC.[147]

The subset of donor-lymphocytes involved in the GVL effect is not entirely defined. Both $CD4^+$ and $CD8^+$ GVL effectors have been described in animal models. Recent studies in man suggest a prominent role of $CD8^+$ cells in acute leukemia and $CD4^+$ cells in CML.[148] The therapeutic index of this approach may be increased by treating the donor lymphocytes, previously activated with recipient PHA-stimulated blast, with anti-CD25 ricin-conjugated antibodies.[149] This procedure gives origin to a CTL population which retains over 75% of its antileukemic activity with only 10% of the initial responsiveness against the non-leukemic cells of the recipient.

Strategies for generation and expansion of specific CTL

A very attractive system for generating and expanding CTL is based on the selection and isolation of tumor-specific peptides (e.g. those encoded by bcr-abl or PML-RARα fusion genes) and to presenting them to T-cells to stimulate a specific T-cell response. The responding T-clones can then be amplified and selected by limiting dilution techniques. Unfortunately, in many cases the tumor-specific peptide is not presented by leukemic cells making the generation of peptide-specific CTL useless.[150] In this regard, the

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transfection in tumor cells of DNA sequences encoding for co-stimulatory molecules (B7-1) or cytokines such as GM-CSF has greatly enhanced the anti-tumor response of T-cells. Alternatively, the use of professional antigen presenting cells (APC), primed with tumor specific antigens (e.g. tumor specific idiotype in low-grade B-cell lymphoma) has proved to be effective for the generation of tumor-specific CTL clones *in vivo* capable of inducing a measurable anti-tumor response.[151] Allo-CTL have also been used against EBV-related lymphoma developed in allograft recipients and HIV patients. EBV infection is controlled in normal individuals by specific CTL which lyse EBV-infected B-cells upon recognition of viral peptides presented on the cell membrane in association with MHC class I molecules.

EBV-specific CTL have been isolated from normal donor leucocytes and expanded *ex vivo* by Rooney *et al*.[152] Following the reinfusion of 1.2×10^8 CTL/m^2 into an allograft recipient, the complete resolution of an EBV-related immunoblastic lymphoma was observed.

Ex vivo expanded CTL can also be used to restore CMV-specific responses in immunodeficient individuals receiving allogeneic BMT. Walter *et al.*[153] treated 14 patients with infusions of $CD8^+$ CTL directed to CMV proteins obtained from bone marrow donors. In this study, CMV-specific CTL were expanded by stimulation with anti-CD3 antibodies coupled with autologous CMV-infected fibroblasts in IL-2-containing culture. This approach to adoptive immunotherapy was well tolerated by the recipients and not associated with severe GVHD.

Future directions

Preliminary clinical data suggest that the efficacy of donor BC infusion for the treatment of leukemic relapse can be significantly improved by the administration of IL-2 *in vivo* after reinfusion and by a brief incubation of BC with IL-2 before the reinfusion.[154] The antitumor efficacy of T-lymphocytes can also be enhanced by transfection of cytokine genes or new receptors. An interesting approach is represented by the binding of TCR to a specific anti-tumor antibody Fab fragment or the use of bispecific (anti-tumor and anti-CD3) antibodies capable of recruiting and expanding tumor-specific CTL at the tumor site.[155] Recently, a significant autologous GVHD effect has been obtained by the addition of γ-IFN to cyclosporin-A in order to upregulate MHC class II molecules .[113] Alternatively, GM-CSF seems to enhance the anti-tumor response by stimulating professional APC (see below).[156]

In conclusion, there is clear evidence that CTL exert their cytotoxic effect through the recognition of minor HLA antigens.[157] However, in some patients a very low frequency of specific antileukemic CTL, responsible for a GVL effect distinct from GVHD, have been isolated. Moreover, genetic approaches, such as the transduction of donor lymphocytes with a suicide gene, have been proposed to control GVHD occurring after CTL administration.[158]

Ex vivo generation of human dendritic APC

Clinical investigators are keenly interested in the role of APC in the initiation of immune responses because of the potential to exploit these cells for immunotherapy of cancer and viral diseases. Pioneer studies in mammals by Steinman *et al*.[159] have demonstrated that the specialized system of APC is constituted by BM-derived dendritic cells (DC). DC are distinguished by their unique potency and ability to capture, process, and present antigens into peptide-HLA complexes to naive T lymphocytes and to deliver the co-stimulatory signals necessary for T lymphocyte activation and proliferation.

Here, we summarize the main experimental evidence supporting the working hypothesis that individuals vaccinated with DC expanded *ex vivo* and engineered to present tumor associated antigen(s) can mount tumor-specific humoral and cellular responses. This can lead to tumor regression as well as protective immunity against tumor growth *in vivo*.[159-162]

Identification of dendritic cells

DC are leukocytes derived from hematopoietic stem cells along the myeloid differentiation pathway (Figure 5). The differentiation of DC is a stepwise process:[163] originating from myeloid progenitors in the BM, *immature DC* distribute via blood to tissues where they have the capacity to take up and to process antigens. As migratory DC, they transit through the lymph or blood to lymphoid organs, where they become *mature DC*, which lose antigen-processing ability and acquire superior antigen-presenting capacity for T lymphocytes.[163] In humans, DC at different developmental stages circulate in PB and they are found in virtually all tissues of the body where, depending on the location, they are referred to as *interstitial DC* (heart, kidney, gut, and lung), *Langerhans cells* (skin, mucous membranes), *interdigitating DC* (thymic medulla, secondary lymphoid tissue); or *veiled cells* (lymph, blood).[163] DC are regarded as distinct from monocytes/macrophages, although they share a common progenitor after the CFU-GM stage. However, this has been questioned as mixed colonies of dendritic cells and macrophages are generated *in vitro* from single $CD34^+$ hematopoietic progenitors more commonly[163-175] than pure DC colonies.[176]

DC can be distinguished from other APC by a) morphology; b) cell-surface membrane phenotype; and c) the strong capacity to present antigens to T cells, usually assessed in the allogeneic mixed leukocyte culture (reviewed in ref. #163).

Cutaneous DC, as well as most of the DC generated *ex vivo* from human $CD34^+$ progenitors cells express high levels of the surface membrane CD1a antigen (Figure 6). Although CD1a antigen can be found on cortical thymocytes and some B lymphocytes, its pres-

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ence (noted by immunofluorescence and flow cytometry) is the most useful way to quantify the *ex vivo* generation of DC from early precursors. In addition to the CD1a antigen, DC express peculiarly high levels of class I and class II histocompatibility complex structures, co-stimulatory molecules for T-lymphocytes such as B7-1 (CD80) and B7-2 (CD86), and adhesion molecules such as ICAM-1 (CD54) and ICAM-3 (CD50) which are involved in DC-dependent T-lymphocyte proliferation. DC lack monocyte/macrophage- and lymphocyte-lineage-restricted antigens with the exception of the CD4 antigen.[170] As shown in Table 3, relevant co-stimulatory (B7-1 and B7-2) and adhesion molecules (ICAM-1) are expressed on all $CD1a^+$ cells derived from $CD34^+$ progenitors, but on fewer $CD1a^+$ cells derived from monocytes.[177,178] More recently, the CD83 cell surface antigen has been recognized as a valuable tool for detecting blood DC.[179]

Ex vivo expansion of dendritic cells

Although DC circulate in the PB and are found in virtually all tissues of the body, it is difficult to obtain enough cells for *ex vivo* manipulation because of their scattered locations and low number in the blood where they account for approximately 0.1% of all leukocytes.[163] For this reason, it has been of crucial interest to know that: a) TNF-α cooperates with GM-CSF to generate DC from $CD34^+$ hematopoietic progenitors from BM, cord blood or PB;[166, 168-176] and b) IL-4 cooperates with GM-CSF in the development of DC from circulating monocytes.[169,180] A detailed description of the methods utilized to obtain human DC from myeloid precursors has been recently reported.[163] However, in evaluating these methods in view of a clinical trial, at least three issues should be taken into consideration:

a) the type of DC generated either from monocytes (monocyte-derived DCs) or from $CD34^+$ hematopoietic progenitors ($CD34^+$ derived DC);
b) the source of serum for DC growth in culture;
c) the combination of cytokines required for optimal *ex vivo* expansion of functional immunostimulatory DC.

Monocyte-derived DC are being employed in patients with advanced stage malignancies in phase I-II clinical trials. The trials are particularly aimed at evaluating toxicity and immune responses after subcutaneous administration following DC pulsing *ex vivo* with either melanoma tumor-associated peptides[181-183] or with B-cell lymphoma and myeloma idiotype proteins from autologous serum.[151,161] Early reports from clinical studies, in patients with melanoma who are HLA-A1 positive and whose malignant cells express the MAGE-1 gene, show that *in vivo* immunization with autologous monocyte-derived DC pulsed with MAGE-1 gene coded nonapeptide, is not toxic and can induce peptide-specific autologous melanoma reactive $CD8^+$ cytotoxic T-lymphocyte responses *in situ* at the vaccination site and at distant tumor sites[181] as well as in PB.[182] From a technical point of view, in these studies the generation of DC from PB mononuclear cells is dependent on a culture medium necessarily containing GM-CSF without serum or with human pooled donor serum. Under these conditions the production of DC is quite scarce in comparison with that achieved with fetal calf serum, as reported

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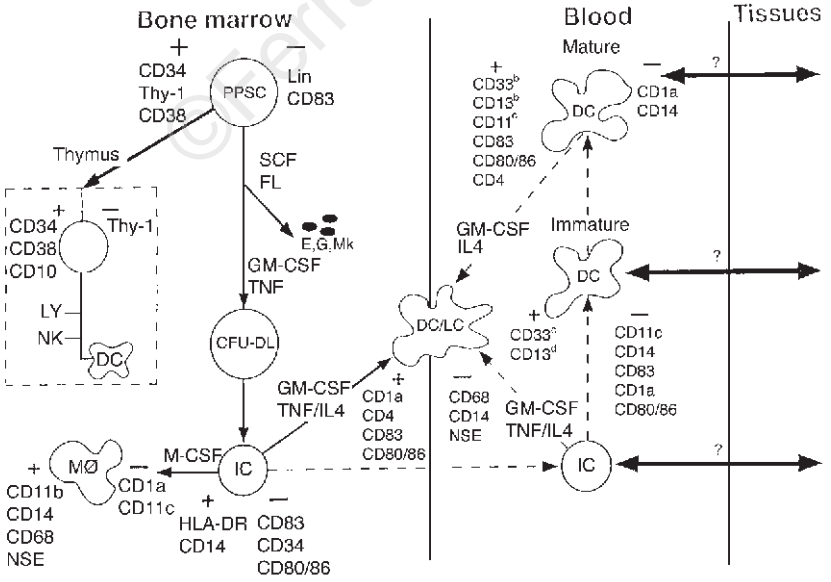

Figure 5. Scheme of DC development in BM, PB and thymus.
Abbreviations: ly, lymphocyte; NK, natural killer cells; NSE, non-specific esterase; PPSC, pluripotent stem cells; CFU-DL, dendritic/Langherans cell colony forming unit. Modified from Reid CA, Br J Haematol 1997; 96:217-33.

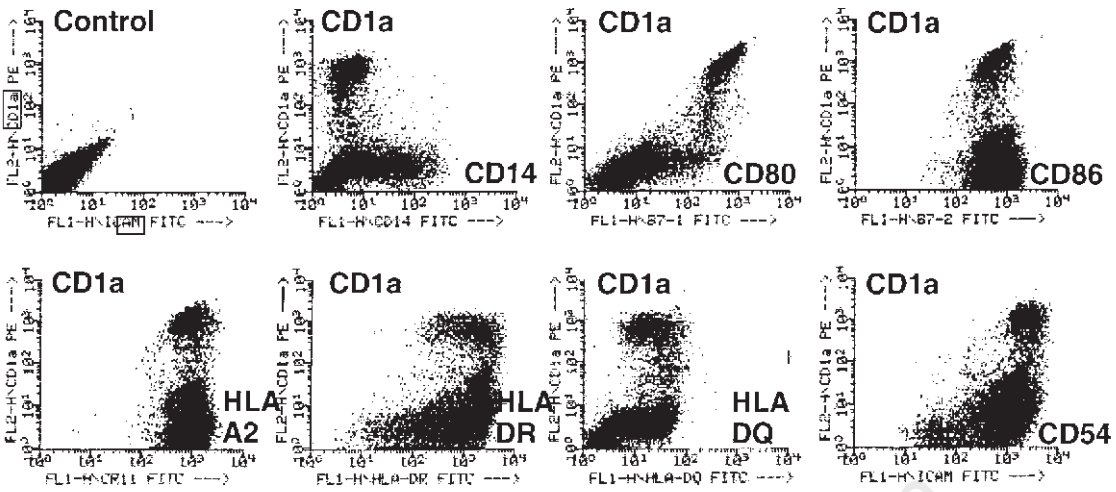

Figure 6. Flow cytometry evaluation of cell surface phenotype of DC derived *ex vivo* from CD34⁺ progenitors on day 12 of culture in the presence of GM-CSF, TNF-α, SCF and FL.

in early studies.[169] However, the presence of fetal calf serum in the culture medium induces undesired DC-mediated immune responses to xenogenic proteins as observed in murine[184] and human preclinical studies.[170] In this regard, experimental data suggest that CD34⁺ cell-derived DC can also be generated in the absence of serum if the culture medium containing GM-CSF and TNF-α is supplemented with TGF-β1.[185]

Modalities for the large-scale procurement of functional DC from CD34⁺ hematopoietic progenitors in patients with cancer have been evaluated.[170] It was found that mobilized PB progenitors currently utilized in phase III trials[186] include a fraction of CD34⁺ DC precursors which give origin *ex vivo* to a progeny with the characteristics of professional APC i.e., typical DC morphology and immunophenotype undistinguishable from cutaneous Langerhans cells and DC from cord blood and BM CD34⁺ cells. Most importantly, these *ex vivo* generated DC retained the capacity to process and present antigens to T lymphocytes as demonstrated by elicitation of HLA class II and class I-restricted activation of CD4⁺ and CD8⁺ autologous T lymphocytes in response to xenogenic antigens of fetal calf serum[170] or melanoma tumor-associated antigen peptides,[177,178] respectively. Quantification of progenitors of DC by limiting dilution analysis of CD34⁺ cells sorted from blood cell autografts showed that they are approximately 140-fold more numerous than in steady-state control autograft. To obtain this favorable result, blood cell autografts were collected at the time of maximal mobilization of CD34⁺ cells into PB as occurs after treatment with high-dose cyclophosphamide and cytokines.

In a systematic search for culture conditions capable of ameliorating the *ex vivo* generation of DC dendritic cells, a variety of exogenous stimuli have been evaluated as well as monocyte-derived versus CD34⁺ cell-derived DC.[170,177,178] In this respect, it has been shown that GM-CSF plus TNF-α-dependent generation of DC from mobilized CD34⁺ cells is 2.5 fold enhanced by either FL or SCF, and 5-fold enhanced by a combination of these growth factors. In addition, autologous high-dose chemotherapy recovery phase serum rather than fetal calf serum or human donor pooled AB serum has been shown to be the optimal serum for the generation of DC. Regardless of the precise mechanism of action of FL and SCF in association with GM-CSF and TNF-α on the enhancement of DC

Table 3. Phenotype of CD1a⁺ dendritic cells generated from mobilized CD34⁺ progenitors or from monocytes.

Antigens	*CD1a⁺ cells derived from* CD34⁺ progenitors	*Monocytes*
CD14	2%	3%
CD80	100%	84%
CD86	100%	67%
CD54	100%	67%
MHC Class I		
HLA-A0201	100%	100%
MHC Class II		
HLA-DR	100%	100%
HLADQ	60%	89%

Modified from Mortarini et al., Cancer Res, in press.

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differentiation and proliferation, these findings have provided new advantageous tools for the large-scale generation of DC from mobilized CD34+ cells in patients undergoing cancer treatment. In fact, the stimulation of CD34+ cells from an average blood cell autograft should permit the generation of a median of 0.6×10^9/kg DC from an average 65 kg individual, i.e., almost 40×10^9 DC. In contrast, differentiation of DC from monocytes in the presence of autologous high-dose chemotherapy recovery phase serum plus GM-CSF and IL-4 is not associated with a comparably high outgrowth of DC.[177,178] These observations, together with the weaker expression of co-stimulatory molecules in monocyte-derived DC in comparison with CD34+ cell-derived DC[187] may favor the utilization of the latter source of APC for the development of active immunization programs involving DC in humans. The comparative efficiency as APC of DC derived from monocytes versus CD34+ hematopoietic progenitors has recently been studied with DC isolated from blood of patients with melanoma. In particular, it has been shown that DC derived from G-CSF-mobilized CD34+ cells are more efficient than those derived from monocytes in inducing melanoma tumor-associated antigen peptide specific activation of autologous CD8+ cytotoxic T-lymphocytes. Interestingly, in the same experiments the latter cells were also capable of lysing a panel of melanoma cell lines sharing the same HLA class I alleles with the patients from whom CD8+ cytotoxic T-lymphocytes were generated with tumor-associated antigen peptide pulsed autologous DC.[177,178] Moreover, CD34+ cells mobilized into PB by G-CSF were shown to be capable of generating a higher number of mature and fully functional DC than their BM counterparts.[188] However, it should be pointed out that, at present, clinical studies utilizing DC as vehicles for anti-tumor vaccination have been carried out with monocyte-derived DC either freshly isolated from PB[152] or cultured *ex vivo*.[161, 181-183] In addition, culture conditions which allow the large scale production of terminally differentiated and fully functional monocyte-derived DC have recently been described.[189]

Dendritic cells for antitumor cell therapy

An extensive review on the clinical use of DC is beyond the scope of this paper, however, a few remarks in this regard are needed.

The goal of vaccination is the induction of protective immunity. Originally, vaccinations were used in the setting of infectious diseases, but are now expanding to include the treatment of allergy, autoimmune diseases, and tumors. A rational approach to vaccination involves 3 steps: a) the identification of the protective effector mechanisms, b) the choice of an antigen that can induce a response in all individuals, and c) the use of an appropriate way to deliver the vaccine to induce the proper type of response.[159-162]

It has now been demonstrated that certain tumor cells are antigenic by expressing tumor-associated antigens that can be recognized by T lymphocytes in a syngeneic host. However, they are often poorly immunogenic, at least in part because they lack the cellular armamentarium for specific T-lymphocyte recognition, activation, and co-stimulation typical of APC especially DC.[190,191] Different mechanisms may account for the ability of tumor cells to evade immune responses. Tumor cells may display low immunogenicity through low MHC and/or tumor-speciifc antigen expression or may downregulate Fas and constitutively express Fas-Ligand, which binds to Fas on cytotoxic T-lymphocytes, resulting in apoptosis of the latter.[192] Furthermore, it has recently been shown that vascular endothelial growth factor produced by tumors inhibits the functional maturation of CD34+ cell-derived DC.[193] When considering the use of the unique antigen-presenting capacity of DC to prime specific antitumor T-lymphocytes, this phenomenon should be taken into account, as it may result in poor recovery and function of DC directly recovered from the blood of cancer patients. In contrast, dendritic APC expanded *ex vivo* in the presence of cytokines and in the absence of inhibitory factors released by tumors would probably be functional.[193-195] However, it should also be considered that published data demonstrate that whereas *in vivo* administration of DC loaded with low doses of tumor antigen enhances antitumor immunity, DC pulsed with high doses of antigen or high numbers of tumor antigen-pulsed DC may inhibit development of immunity.[196] This finding supports the notion that stimulation of DC-mediated antigen presentation in vivo may act in a *tolerogenic* or immunogenic fashion depending on a variety of partially understood factors.[197]

Basically, there are at least two approaches to tumor vaccination (Figure 7). The first is to identify a tumor-associated antigen to be used as a vaccine, the second is to increase the immunogenicity of tumor cells and let the immune system decide which antigen to target. Indeed, in experimental models with the appropriate manipulation exploiting the physiologic function of antigen-presenting DC, the immune system can be induced to mount responses that can kill tumor cells and also protect animals from subsequent challenge even with a poorly immunogenic tumor.[185,198-203]

Given the richness of recently identified tumor associated antigens and their corresponding peptide epitopes recognized by MHC-restricted CD8+ or CD4+ T lymphocytes (Table 4), investigators are currently evaluating the clinical efficacy of specific tumor-associated antigen-based vaccines for the treatment of various malignancies. Recently, in a cooperative clinical trial it was observed that partial tumor regressions can occur in HLA-A1+ patients with melanoma treated with a naked MAGE 3 peptide epitope vaccine even in the absence of any engineering of antigen-presenting cells or adjuvant cytokine(s).[204] This clinical evidence induces the belief that the effective-

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ness of peptide-based vaccines is likely to benefit further from administration of appropriate cytokines[156,205,206] or cellular adjuvants (e.g. DC) capable of promoting cellular immunity.

Among hematologic malignancies, CML is being intensively evaluated as a possible target of dendritic APC-based immunotherapy. It is well known that CML is characterized by a specific translocation of the *c-abl* oncogene (9q34) to the *bcr* region on chromosome 22 (22q11). Alternative recombination sites involving either the second or third exon of the *bcr* gene splicing to exon 2 of the *abl* gene yield two potential fusion gene transcripts, b2a2 and b3a2, respectively. The translated 210-kd *bcr-abl* fusion protein, which has abnormal tyrosine kinase activity, includes a new potentially antigenic sequence at the fusion site: a new amino acid is generated at the junctional site by the fusion event; in the b2a2 fusion a glutamic acid (E) is encoded, whereas in the b3a2 recombination event a lysine (K) is generated. Interestingly, a *bcr-abl* peptide from the b3a2 fusion region has been found to be immunogenic in mice.[207] In humans, binding of b3a2 peptides to various HLA class I alleles[208] and priming of $CD8^+$ cytotoxic T-lymphocytes *in vitro* has been described although the capacity of these peptide-specific $CD8^+$ T-lymphocytes to lyse CML cells has not been determined.[209] In contrast, in a recent study it has been demonstrated that $CD4^+$ T-lymphocytes can be identified that proliferate in an HLA class-II restricted manner in response to a 11mer (GFKQSSKALQR) b3a2 peptide especially when the latter is presented by purified $CMRF\text{-}44^+$ blood DC.[210] In the same study the peptide-specific $CD4^+$ T-lymphocytes were able to respond to the whole protein in crude extract from CML cells. Intriguingly, dendritic antigen-presenting cells in CML patients can be derived from the malignant clone and these malignant dendritic cells can induce antileukemic reactivity in autologous T lymphocytes without the necessity of additional exogenous antigens.[211]

Although, the above observations cannot be extrapolated *a priori* to other malignancies carrying specific translocations and corresponding fusion genes and products,[212] further investigations on the possible clinical application of *bcr-abl* peptide(s) presented by autologous dendritic antigen-presenting cells are warranted.

The translation into the clinical setting of the above experimental hints favoring the use of DC pulsed *ex vivo* with synthetic tumor-associated antigen peptides for effective cell therapy in humans is likely to be hampered by: a) the limited availability of patients with HLA typing compatible for the utilized tumor-associated antigen peptide(s); b) the occurrence of independent mechanisms of tumor escape *in vivo* such as loss of expression of tumor-associated antigens or of HLA class I during tumor progression; and c) the short duration of the immune responses thus requiring annoying boost vaccinations. It has been suggested[213] that these limitations may be overcome by transduction of genes encoding relevant proteins into DC or their progenitors so that DC could tailor peptides to their own HLA molecules thus obviating the need to synthesize tumor-specific peptides most of which have stringent HLA restrictions. A further advantage of the transduction approach may be the stable long-term expression of the antigen by the DC, which would allow its presentation to the immune system for longer periods without the concerns about the turnover of preformed peptide/HLA complexes *in vivo* after immunization.[214]

Based on the above experimental body of evidence, a pioneer clinical trial has evaluated the ability of autologous monocyte-derived DC pulsed *ex vivo* with non Hodgkin's lymphoma-specific idiotype protein

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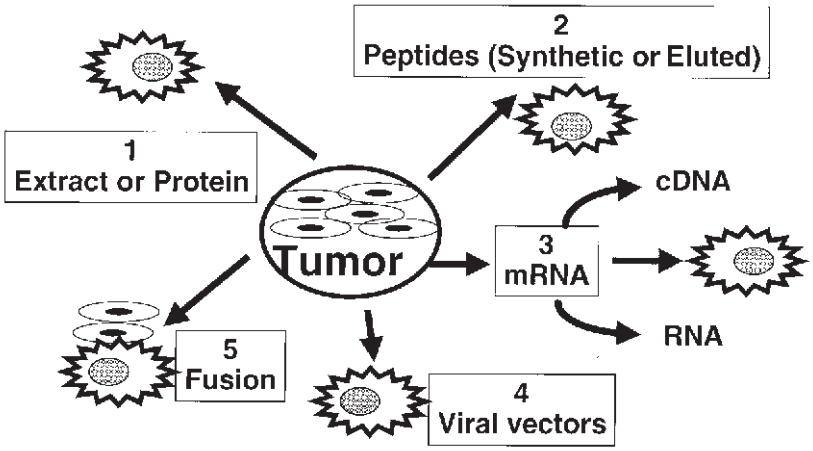

Figure 7. Sources of tumor antigens for DC-based cancer vaccines.

Table 4. Tumor antigens capable of eliciting T-lymphocyte responses.

Activated oncogene products	
Mutated:	Position 12 point mutation of 21^{ras}
Rearranged:	bcr-abl (b3a2 peptide)
Overexpressed:	HER-2/neu
Tumor suppressor gene products	
	p53 mutations
Reactivated embryonic gene products	
	MAGE family (at least 12 genes)
	BAGE
	GAGE
Melanocyte differentiation antigens	
	Tyrosinase protein
	Melan-A/MART1
	gp100
	gp75
Viral gene products	
	Human papilloma virus antigens (E6, E7)
	EBV EBNA-1 gene products
Idiotype epitopes	
	Ig and TCR hypervariable regions

to stimulate host immunity when infused as a vaccine.[151] In this study active immunotherapy of patients with B-cell lymphoma against idiotypic determinants led to anti-tumor immunity that correlated with improved clinical outcome in some patients.

Regardless of the type of cell manipulation (Figure 7) (*ex vivo* pulse with tumor-associated antigen peptides versus transduction with tumor associated antigen genes versus immunization with fusions of dendritic and carcinoma cells[215]) that will be successful in clinical applications the necessity of methods of generating large numbers of functional DC is implicit for the evolution of such studies.

Future directions

Since DC have been shown to be intimately involved in the generation of $CD4^+$ and $CD8^+$ T-lymphocyte mediated tumor-specific immunity, it is attractive to speculate that vaccination with DC pulsed or engineered *ex vivo* to present tumor antigen(s) may be effective in generating tumor immunity *in vivo*. Among the recently prospected sources of DC, namely BM, neonatal cord blood, and adult PB, the last is certainly the richest and most accessible in all patients with cancer, although it remains to be confirmed whether functional differences will favor the utilization of monocyte- versus $CD34^+$ cell-derived DC. Thus, in the clinical setting of adoptive immunotherapy for patients with malignancies, a therapeutic protocol could be envisioned involving the mobilization of $CD34^+$ cells into PB with hematopoietic growth factors, with or without prior intensive chemotherapy. Thus, enrichment of hematopoietic progenitor cells could be followed by *ex vivo* generation of DC pulsed or engineered to present tumor antigen(s), to be reinfused as a potential secondary tumor-specific immunotherapy or vaccination. It remains to be established whether the latter effect could be further enhanced by *in vivo* adjuvant cytokines such as GM-CSF and/or FL, as occurs in murine models.

A potential advantage of *ex vivo* immune cell therapy over direct *in vivo* immune intervention, is the lack of functional inhibition that may occur *in vivo*. This hypothesis is based on a recently proposed mechanism of tumor escape/resistance from the host immune system, in which cancer cells produce a vascular endothelial growth factor that impairs antigen presentation required to induce specific antitumor immune responses *in vivo*.[193]

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All the authors equally contributed to the manuscript and they are listed in alphabetical order.

Disclosures

Conflict of interest. This review article was prepared by a group of experts designated by Haematologica and by representatives of two pharmaceutical companies, Amgen Italia SpA and Dompé Biotec SpA, both from Milan, Italy. This co-operation between a medical journal and pharmaceutical companies is based on the common aim of achieving optimal use of new therapeutic procedures in medical practice. In agreement with the Journal's Conflict of Interest policy, the reader is given the following information. The preparation of this manuscript was supported by educational grants from the two companies. Dompé Biotec SpA sells G-CSF and rHuEpo in Italy, and Amgen Italia SpA has a stake in Dompé Biotec SpA.

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