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Engineering Humanized Mice for Improved Hematopoietic Reconstitution

Running Title: Improving Humanized Mice

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Abstract
Humanized mice are immunodeficient mice engrafted with human hematopoietic stem cells that give rise to various lineages of human blood cells throughout the life of the animal. This article reviews recent advances in the generation of humanized mice, focusing on practical considerations. We discuss features of different immunodeficient recipient mouse strains, sources of human hematopoietic stem cells, advances in expansion and genetic modification of hematopoietic stem cells, and techniques to modulate the cytokine environment of recipient mice in order to enhance reconstitution of specific human blood lineage cells. We highlight the opportunities created by new technologies and discuss practical considerations on how to make best use of the widening array of basic models for specific research applications.

Introduction
In recent years there has been an explosion of interest in reconstituting the human immune system in mice to create humanized mice or 'humice'. The critical technical breakthrough was introduction of IL2Rγ [or the common gamma chain (γc)] ablation onto already immune compromised backgrounds to produce receptive recipient mice lacking endogenous T, B and natural killer (NK) cells (1-4). In this review we consider the steps involved in constructing humanized mice in these animals by injecting human hematopoietic stem cells (HSCs) to reconstitute a human immune system. We do not consider the more complex humanized mouse models where multiple tissues are implanted (for example to create a human thymic environment or reconstitute multiple human tissues), these sophisticated models are discussed by Mamoro Ito in another review in this issue. We also do not consider the models developed using differentiated or cancerous cells, though those using peripheral blood are reviewed by Brehm and Shultz elsewhere in this issue.

We review recent advances in three areas of humanized mouse technology. First, we discuss different strains of recipient mice and how they improve the durability or quality of the human graft. Second, we discuss sources of human HSCs, and methods to culture and genetically modify HSCs following purification. Third, we review techniques to alter the cytokine environment once a graft has been established. While progress has been made in each of these areas they have largely been worked on in isolation. We discuss outstanding questions and the opportunities of combining these technologies at the end of this review. At each stage we summarize the literature and discuss the advantages and drawbacks of particular strategies based on our own and other's experience.

Selection of Mouse Strains
The 'modern' humanized mouse was created in the past decade by three independent introductions of γc mutations into already immune compromised strains: Creating the NOD Shi-SCID γc^{null} (NOG) (1), NOD Itz-SCID $\gamma c^{-/-}$ (NSG) (4) and Balb/c $Rag^{-/-}$ $\gamma c^{-/-}$ (2) mouse strains. In a recent comparison of mouse strains, female NSG mice were shown to be the best recipients for small numbers of human HSCs, based on engraftment efficiency (5). However, when large

numbers of HSCs are transferred, the difference largely disappears and very robust engraftment can be sustained in all three strains of recipient mice.

These first generation 'modern' humice provide an important tool to study infection by human pathogens, especially those that infect human blood lineage cells. They have also allowed the investigation of human immune responses to pathogens in a small animal model. However, in mouse recipients the number and diversity of human cells are still below normal human ranges. Additionally, studies suggest that some human cells are not fully functional in mice (6, 7).

This has led to the current focus on developing new genetic models that provide a better and more human environment for the engraftment of HSCs and the development and function of their progeny. These models divide into two categories – replacing the mouse-secreted factors with their human counterparts and improving direct cell-cell or receptor-ligand interactions. In addition, further work has been done to reduce rejection of human HSCs in humanized mice.

Soluble factors that enhance reconstitution

Cytokines, chemokines and growth factors comprise the important families of (generally) small secreted proteins important for cell development, differentiation, trafficking and survival. Many of them have been found to exhibit significant sequence divergence between human and mouse and to lack functional cross-reactivity *in vitro*. This leads to the lack of critical human species-specific signals that support human cell survival, development and function in mice. The myeloid cell lineages are worst effected as key factors seem to be almost wholly incompatible. As a result Billerbeck, *et al.* created NSG mice transgenically expressing human stem cell factor (SCF), granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-3 (NSG-SGM3). Transplantation of human $CD34^+$ fetal liver cells into these mice led to a limited improvement of total human hematopoietic cell reconstitution. Although the frequencies were elevated, human myeloid cells were still mostly found in the bone marrow. The most notable improvement was an increase in the number and functions of $CD4^+FoxP3^+$ human regulatory T cells (8).

Some other groups specifically replaced mouse cytokine genes with the corresponding human cytokine genes. Homozygous knock-in of the thrombopoietin (TPO) gene in Balb/c $Rag2^{-/-}$ $\gamma c^{-/-}$ mice led to increased levels of reconstitution of human $CD45^+$ cells from 40% to 80% and an improved ratio of myelomonocytic versus lymphoid lineage cells from 14% to 32% of human $CD45^+$ cells in the bone marrow of the recipients (9). Moreover, it was also shown that human stem and progenitor cells were better maintained in the bone marrow of these TPO knock-in mice. In comparison to the improvement restricted in bone marrow in TPO knock-in mice, the same group also generated human GM-CSF/IL-3 knock-in mice in order to improve the development and function of human myeloid cells. They showed that after engraftment with human cord blood (CB) $CD34^+$ cells, GM-CSF/IL-3 knock-in mice had improved human myeloid cell reconstitution, particularly alveolar macrophages in the lung. When challenged by influenza virus, the humanized mice exhibited some levels of human innate immune responses (10). Another group generated macrophage colony-stimulating factor (M-CSF) knock-in Balb/c $Rag2^{-/-}$ $\gamma c^{-/-}$ mice for supporting monocyte/macrophage lineage development. They reported more

efficient differentiation and enhanced frequencies of human $CD33^+CD14^+$ monocytes/macrophages not only in the bone marrow but also in the spleen, peripheral blood, lung, liver and peritoneal cavity (11).

Cell-cell interactions

Given the known MHC mismatch issues with human T cell interactions in humanized mice (this is indeed the main reason for the complex thymic grafts carried out by many laboratories) one of the main areas of improvement has been in engineering mice to express human major histocompatibility complex (MHC) genes. HLA-A2 transgenic mice expressing the most common human MHC I gene (~40% allelic frequency) have been reported recently to display enhanced CD8 T cell function (12, 13). Recent studies report that engraftment of NSG HLA-DR4 transgenic mice with matching human HSCs produces all subsets of immunoglobulins at levels found in humans and improved CD4 T cell function (14). Mice lacking the murine MHC II gene and transgenic for HLA-DR4 have been produced, which should eliminate the diabetogenic and unusual NOD $I\text{-}A^{g7}$, though as yet no report is available regarding their reconstitution with human HSCs (15).

Reduced rejection

NSG, NOG, and Balb/c $Rag^{-/-}$ $\gamma c^{-/-}$ mice all lack T, B and NK cells, which greatly reduces their ability to reject grafts. However, human cells can still be rejected by mouse macrophages. One significant difference between the NOD and Balb/c mouse strains is a polymorphism in SIRPα, allowing the NOD SIRPα to recognize human CD47 and therefore inhibit macrophage phagocytosis (16). The CD47-SIRPα interaction has since been shown to be critical to the survival of circulating HSCs and has become a major therapeutic target in hematologic cancers (17-19). The Flavell group addressed the drawback in the Balb/c mouse strain by humanizing the SIRPα and were able to demonstrate that this not only allows Balb/c mice to reconstitute to levels similar to that seen in NSG mice but also improves immune function (20).

Summary

The proliferation of strains of mice for reconstitution in recent years has made the choice of which strain to use difficult. Given that the field is currently in flux with the many incremental improvements being bred together, it is difficult to predict which strain(s) will become more widely adopted. However, as many of these mouse strains are not at present commercially available, this can limit the options. In addition, many highly modified mouse strains breed poorly. The robust breeding characteristic and ease of access to NSG mice mean that for the time being NSG mice are still the most attractive recipients available. If a study requires one of the recently added human genes care should be taken to establish that the mice breed robustly as this has a major impact on both the cost and timeliness with which a study can be completed.

Choice of HSCs

Humanized mouse researchers use the results of the active fields of stem cell biology and transplantation medicine to help select which cells to inject. In stem cell research long-term reconstitution of immune compromised mice has been used to define all the sources of human HSCs. However, injection of large numbers of stem cells to produce well-reconstituted animals

is more like clinical transplantation than the delicate fractionation typical of stem cell research. The critical parameters for humanized mouse research are 1) the aggregate reconstitution potential and 2) the lack of mature T and B cells in the donor cell population that mediate graft versus host disease. As a result, the definitive markers of the most primitive human HSCs proposed in recent years (21, 22) are less important than less discriminating markers such as CD34 and CD133, which simply capture the bulk of the long term reconstituting activity (23-25). Using these markers (either singly or in combination) one can fractionate cells and make reproducible decisions about how many cells are needed per animal to ensure optimal engraftment for a particular study. The three main sources of HSCs are mobilized adult peripheral blood, umbilical cord blood and fetal liver. We review the pros and cons of each briefly here. One decision common to all HSCs is whether to use fresh or frozen cells. In either case cells should be assayed directly before injection for reproducible results. In general freshly prepared HSCs reconstitute better as there are no losses from freeze and thaw. Although freezing cells makes the timing of reconstitution easier cell viability is effected and optimizing freeze/thaw procedures is critical. Injection timing is particularly important when using newborn pups as recipients (a popular choice as fewer cells are needed to reconstitute each mouse). We try to use fresh cells and if this isn't possible prefer to allow cells to recover from thawing overnight before injection, while precise methods vary from lab to lab it is important to be consistent and assess how many cells are needed to achieve good reconstitution with the selected protocol.

Mobilized adult HSCs

This is the most abundant source of human $CD34^+$ HSCs but unfortunately the least effective at reconstituting mice. While large numbers of cells are available collecting them requires relatively invasive procedures such as GM-CSF mobilization followed by leukaphoresis or bone marrow biopsy. The quality of reconstitution varies based on donor age and other clinical factors. There have been relatively few studies which have used these cells to date: Shultz injected 700,000 mobilized $CD34^+$ cells and observed ~6% chimerism in the peripheral blood of adult NSG recipients (4). Matsumura and co-workers injected an average of ~1.1 million mobilized $CD34^+$ cells in NOG mice and observed with an average reconstitution of ~11% in the blood (26). Hayakawa et al. injected 2 million $CD34^+$ cells from adult blood achieving ~16% reconstitution in the peripheral blood of the recipient NSG mice with Busulfan conditioning (27). As both Matsumura and Hayakawa also injected CB $CD34^+$ cells in the same studies and observed far better engraftment (47% and 65%, respectively), it is clear that adult HSCs engraft with lower efficiency than cells from younger donors. These studies suggest that creating highly chimeric animals using mobilized peripheral blood stem cells is likely to be inefficient. Due to the invasive nature of the procedures to harvest the cells, it is difficult to justify ethically in the face of less invasive alternatives. One major advantage of using HSCs from adult is that it is possible with institutional review board oversight to use information from the medical history of patients to study the genetic contributions to specific diseases. While as yet no study of this type has been reported, this type of research is an area where physician scientists with access to patient cohorts can potentially use humanized mice to create specific clinically relevant models of disease and drug toxicity.

Umbilical cord blood HSCs

Umbilical cord blood (CB) has been a major source of HSCs for the construction of humanized mice. As shown by the Ito group, CB $CD34^+$ cells have many times the reconstituting activity of those in adults (26). Cord blood is easy to obtain (as it is considered medical waste), though unlike adult samples there is no medical history that may inform a particular study. Typically, CB cells are purified by positive selection for a stem cell marker, typically CD34 or CD133, then numbers used in reconstitution are normalized by phenotype, usually $CD34^+$, $CD34^+CD38^-$ or $CD34^+CD133^+$ cells. A typical CB sample yields about 1 million purified $CD34^+$ cells (with a range from 200,000 to 10 million). Reconstitution levels and numbers of cells injected vary from lab to lab and each research team must establish how many cells give optimal engraftment for their research. In general fewer cells are needed to reconstitute new born mice rather than adults. Typical reconstitutions with newborn recipients use ~100,000 $CD34^+$ cells yielding a 50-75% reconstitution (3, 23), with ~15-20% of these cells lacking CD38 (a marker of differentiation) and accounting for most of the reconstituting activity as shown by the similar reconstitution of ~20,000 $CD34^+CD38^-$ cells to 100,000 $CD34^+$ cells (3).

While less efficient than injection of neonates, adult mice are frequently used as recipients due to the ease of injection of cells intravenously and the simplicity of using fresh cells (as timed breeding is not required). Reported levels of reconstitution again vary from lab to lab and with specific recipient mouse strains. Injection of 100,000 to 370,000 CB $CD34^+$ cells yields ~10-25% reconstitution in the peripheral blood of NOG mice (1, 26). Injection of 2 million CB $CD34^+$ cells gives rise to ~65% engraftment in the peripheral blood of NSG mice (27). One interesting recent result from John Dick and colleagues shows that female NSG mice reconstitute better than their male counterparts, especially at low cell doses, showing ~3x increases in bone marrow reconstitution. With the ease of consolidating and rearranging female mice (as they do not fight), this approach makes working with adult females particularly attractive (5).

In addition, minimally purified cord blood cells were used in recent work by Shultz, Greiner and colleagues. Injection of this CB cell population (which only had CD3+ cells depleted) containing ~30,000 $CD34^+$ cells gives rise to robust engraftment (~50% chimerism in the peripheral blood) (13). This technique has not been replicated to date but the presence of large supporting cell populations may enhance engraftment and allow greater numbers of humice to be constructed from each cord blood sample.

Fetal liver HSCs

Fetal liver is also a rich source of primitive human HSCs with high repopulating potential, though again there is no patient specific medical information available to guide research. While fetal liver is disposed of as medical waste the ethical issues associated with its use require more detailed IRB oversight. However it is increasingly popular due to the abundance of cells (a typical sample at 15 to 23 weeks of gestation yields ~80 to 280 million $CD34^+$ cells) with similar per cell reconstitution potential to CB $CD34^+$ cells. Fetal liver has been used extensively for making so called 'BLT' mice due to the advantages of a thymic graft, an area discussed by Mamoru Ito in his review in this issue. Typically, injection of fetal liver HSCs is done in newborn mice, with engraftment potential varying from strain to strain. Balb/c $Rag^{-/-}$ $\gamma c^{-/-}$ mice typically

yield 10-50% chimerism in the peripheral blood when injected with 500,000 fetal liver $CD34^+$ cells (28, 29) while a higher reconstitution (30-90% chimerism in the peripheral blood) can be achieved in NSG mice when injected with 100,000 fetal liver $CD34^+$ cells according to our experience. If the researcher can access fetal liver it offers an attractive source of cells due to the large numbers of $CD34^+$ cells obtainable from a single sample.

Summary

The most broadly accessible sources of human HSCs are umbilical cord blood and fetal liver, with mobilized adult blood both harder to obtain and generally inferior for reconstitution. The growing numbers of sophisticated multi organ models are increasing the popularity of fetal samples as multiple tissues and/or types of stem cells can be harvested from a single donor. The one consistent theme is that with limited exceptions reconstitutions of newborn mice rather than adults have proven more efficient at establishing robust engraftment with limited HSCs. While this limits the procedures that can be used at the time of engraftment the gains made in terms of numbers of mice engrafted (and thus the size of well controlled experiment groups) outweigh the technical difficulties. However research supplies of all of these cell types are limited and a major challenge is to either increase the numbers of mice that can be made from each donation or identify new sources of cells by transdifferentiation or improved techniques to harvest cells from adult donors (where the lower reconstituting activity per cell is offset by far higher cell numbers). A final note is that there is considerable variation in SCID repopulating potential between labs depending on the cell source, handling and other criteria. Therefore, it is critical to begin any study by titrating cells to find the smallest number of cells that gives the desired degree of reconstitution.

Expansion of HSCs

With supplies of human HSCs limited by practical and ethical considerations, one approach is to expand HSCs by culturing *in vitro*. The work done in the field of stem cell research (where the same strains of mice are used for the most rigorous tests of mouse reconstitution as are used for humanization) as well as that of human transplantation offers a number of attractive strategies to expand HSCs. Numerous factors have been identified which alone or more often in combination support the culture and expansion of HSCs and this active field is regularly reviewed (30-32).

It is important to consider the different goals of stem cell research, transplantation and humanized mouse studies when evaluating culture methods to decide which ones are best for making humanized mice. Studies where the primary focus of the investigators was the generation of humanized mice and their detailed characterization following injection of cultured cells are few in number. Our group and that of Dale Greiner are the notable contributors to this area. While these studies include detailed analysis of the lineages of cells produced and reconstitution levels over the timescale used for humanized mouse studies many more studies have focused on stem cell culture methods. Here there are far fewer details of cell phenotype but valuable information can still be acquired. The critical data one needs to assess a culture method are the fold increase in long term SCID repopulating activity, whether reconstitution was multi-lineage and ideally some comparison of the lineages and proportions of cells produced. Many reports written for other fields lack one or more of these pieces of information. Without

evidence of long term *in vivo* repopulating activity, a culture method should be viewed with skepticism.

We review the best culture methods reported to date below and assess their pros and cons, this information is summarized in table 1. These cultures are broadly divided into two categories: feeder free cultures and cultures where another cell type serves as a feeder layer.

Feeder cell free culture

SCF, TPO, Fms-related tyrosine kinase 3 ligand (Flt-3L) culture: This is a well established stem cell culture cocktail which has been characterized in humanized mouse research by the Greiner lab who cultured HSCs and injected 1 million $CD34^+$ cells into adult NSG recipients. The peripheral blood chimerism of ~4% was fairly low given the number of cells injected following culture although the reported 36 fold increase in $CD34^+$ cells indicates this chimerism was achieved with ~30,000 input $CD34^+$ cells. However, the most interesting point was that they saw more robust myeloid reconstitution than usually found in humanized mice and less lymphoid reconstitution (33).

Angiopoietin culture: Culturing human HSCs with angiopoietin-like proteins in combination with SCF, TPO, FGF and IGFBP2 gives an ~20x increase in SCID repopulating activity (measured rigorously by limiting dilution) and results in a robust multi-lineage engraftment which gives 30-60% chimerism in the blood in adult NSG mice with 250,000-500,000 $CD34^+CD133^+$ cultured cells. It also results in the efficient reconstitution of neonate NSG mice (23, 34, 35). This combination of factors results in no particular bias in the cell types produced as reconstituted mice have very similar proportions of cells to those seen with uncultured HSCs. A complete characterization can be found in our recent publication (23).

StemReginin culture: Recent reports that the aryl hydrocarbon antagonist StemReginin 1 (SR1) can enhance HSC culture are of great interest – the chemical is easy to synthesize and a great deal cheaper than the high concentrations of cytokines used to culture HSCs to date. However, it has only been shown to work in the presence of large amounts of cytokines and seems to have no activity alone (36, 37). The ~16x increase in cell numbers in culture is comparable to that seen with angiopoietin like proteins but as yet the resultant cultured cells have not been characterized in nearly as much details as the cells from the angiopoietin cultures.

Notch ligand culture: Immobilized engineered notch ligand $Delta1^{ext\text{-}Ig}$ has been shown by Delaney and co-workers to be a potent enhancer of HSC expansion in culture and has been further shown to have significant benefits in reducing the period of neutropenia following transplantation in humans (38). While the clinical benefits of culture demonstrated here are of great importance the loss of cultured cells over time and the relatively low fold increase in SCID repopulating cells in long term mouse assays (~6x) means that the culture condition is at present not as promising as SR1 or angiopoietin-like 5. However, there is further work indicating that this is a potentially promising area of research as pleiotrophin has been shown by Himburg and co-workers to expand both human and mouse HSCs through PI3 kinase and notch

mediated pathways, although the expansion of human cells was not quantified or extensively characterized *in vivo* (39).

Summary: The existing feeder free culture techniques offer several ways to reach a 10-20 fold increase in SCID repopulating activity. However, to date no effort has been made to combine and optimize the existing distinct cocktails to produce the best culture conditions. It is unknown whether these culture conditions will prove to be additive or even synergistic. Given the relatively superficial (for humanized mouse research) analysis performed on SR1 the easiest culture methods to use without extensive extra characterization are the angiopoietin-like 5 and FLT-3L cultures. The work of Greiner and co-workers has made the cell polarizing effects of FLT-3L quite clear and offer strong evidence that this factor (which was identified as a stem cell expansion factor using NOD/SCID mice which do not have good lymphoid reconstitution (32)) is in fact a myeloid growth and differentiation factor, making its introduction into cultures attractive if the study of myeloid cells is the end goal. The main advantage of myeloid growth factors can be realized either by introducing them into the mouse after reconstitution or doing so in the germ-line (as discussed elsewhere in this review). This leaves the angiopoietin-like 5 culture as both the most effective (20x expansion) and the most fully characterized of the cultures to date. The one caveat with this culture is that angiopoietin-like 5 production is challenging and it suffers from batch and vendor to vendor variation in biological activity, care should be taken to assay each batch in house as at present expansion of human HSCs is not tested by any vendor.

Co-culture

The other technique used in HSC expansion is co-culture with mesenchymal stem cells (MSCs) where very large increases in $CD34^+$ cell numbers have been reported, especially with sophisticated 3D culture techniques. However, the main issue with these culture methods from the perspective of humanized mouse research is that the resultant cells are rarely injected into mice and long-term characterization is even rarer. While some pre-clinical work from the Shpall lab has shown promising results their focus has been neutropenia. They have shown only modest expansion of long term SCID repopulating activity and have directed their efforts towards rapidly producing neutrophils and platelets following engraftment (40).

One paper where MSC co-culture is characterized for humanized mouse work is our study of MSCs and angiopoietin-like 5 (34) where we characterized the cells produced in great detail and were able to demonstrate an ~60x increase in the SCID repopulating cell numbers. The resultant mice demonstrated a T cell bias in their reconstitutions that results in a superior T cell dependent immune response both in terms of antibody titer and reactive T cells to tetanus toxoid. Taken together these data represent the most effective expansion of HSCs to date albeit with a polarization of the cells, which we are presently investigating. For specific studies where a T cell bias is desirable – such as the very active HIV research community – this should prove advantageous. For many other labs where T cell activity is a neutral issue this offers an attractive way forward. However, the technical difficulty of maintaining low passage MSCs for co-culture means that this culture method requires more expertise to run routinely than many other culture methods reported to date.

Summary

HSC cultures developed primarily for stem cell research have offered several extremely attractive methods to increase long term SCID repopulating cell numbers and allow a commensurate increase in the number of mice that can be made from a single sample. We can, with culture, routinely make large groups of adult mice from a single cord blood sample. For most research purposes the feeder cell free cultures with their reduced technical difficulty and lack of cell polarizing effects offer the most attractive way forward with angiopoietin-like 5 culture (~20x expansion) offering the most attractive culture described to date. If enhanced T cell activity is desirable the extra work of doing MSC cultures is offset by a further modest ~3x increase in $CD34^+$ cell numbers (~60x total). One final advantage of an effective culture method is that the time spent *in vitro* allows transduction of the HSCs with lentiviral or retroviral vectors to make genetically modified human leukocytes for comparative studies analogous to the use of knockout and transgenic mice.

Table 1: Summary of the different HSC culture methods characterized in humice to date

Method	Fold increase	Notes	Ref
SCF/TPO/FLT-3L	~3x/21 days	Myeloid bias	(33)
SCF/TPO/FGF/ IGFBP2/Angptl5	~20x/10days	Angiopoietin-like 5 needs to be assayed in house.	(23)
SCF/TPO/IL6/SR1	~15x/21 days	SR1 culture cells poorly characterized in humice to date.	(36)
SCF/FLT-3L/ IL6/TPO/Delta1	~6x/21 days	Expanded cells do not engraft long term in competition with uncultured cells in the clinic.	(38)
SCF/TPO/FGF/ IGFBP2/MSC-Angptl5	~60x/10 days	T cell bias	(34)

Genetic Modification of HSCs

HSC transduction is an active multi-disciplinary area of research and a full review of this field is beyond the scope of this article. While important work has been done recently in the area of *in vivo* transduction (41) and a great deal of active research has studied the transduction of all lineages of cells in both mouse and human, we have focused on the transduction of human HSCs which are then assayed *in vivo* in humanized mice. We have not reviewed the detailed protocols required to produce virus and transduce HSCs, which are a topic for an entire dedicated review. Instead, we focus on two key decisions – the choice of viral vector system and promoter selection and consider their effects on downstream studies.

One of the major barriers to HSC based models is the lack of a robust protocol for selecting transduced HSCs, while work has been done with puromycin selection of cultured cells (42), a rigorous assessment of the maintenance of long term HSC activity has not been undertaken. Until a robust protocol is developed transduction is limited to the ~50% infection rates reported in the literature to date as higher doses of virus tend to kill HSCs. In some systems, this can be an advantage as the transduced population can be compared to internal control populations. In

situations where the presence of wild type cells will interfere with discerning the expected phenotype, a method of isolating transduced cells is necessary.

Choice of viral vector system

In order to mediate long term gene expression *in vivo* there are broadly three choices of viral vector systems: adeno-associated viral vectors (AAV), retroviral vectors and lentiviral vectors.

Adeno-associated viral vectors (AAV): There have been controversies in the efficiency of AAV mediated transfection of human HSCs. It has been reported that AAV2-mediated transgene expression in $CD34^+$ cells is transient, presumably because of inefficient integration (43). Several groups have shown successful transduction of human $CD34^+$ HSCs by recombinant AAV2 vectors (44-48). Paz et al. recently demonstrated that recombinant AAV2 vector integrated more efficiently in the more quiescent sub-populations of human $CD34^+$ HSCs (49). Most interestingly for humanized mouse studies, Santat et al. have documented successful transduction of primitive human $CD34^+$ HSCs capable of serial engraftment in NOD/SCID mice (46). With the availability of several serotypes, rational design of capsid mutants, and strategies (self-complementary vector genomes, hematopoietic cell-specific promoters), it is becoming feasible to achieve efficient transduction of human HSCs by AAV vectors (50).

Retroviral vectors: These vectors are replication defective viral vectors derived from the gamma-retroviruses. They require a dividing cell to successfully infect and generally mediate constitutive gene expression from the viral long terminal repeat (LTR). Retroviral vectors have two major problems for long term HSC work: First, they tend to be silenced over time especially as cells differentiate to produce committed progeny. Second, they tend to disrupt gene expression and have intrinsic oncogenic potential, which was illustrated by the IL2Rγ gene therapy clinical trials where the retroviral integration was in almost identical areas of the genome in the cancers of multiple patients (51-54). However, if the objective of a study is to induce hematologic malignancies this can actually be an advantage as demonstrated by the Dick group in their work to retrovirally induce acute leukemia using MLL-ENL (55).

Lentiviral vectors: Lentiviral vectors derived from HIV-1 are now the favored gene therapy vector with a combination of desirable features – LTRs which do not drive gene expression (56), no direct link to cancer (57), the ability to infect non-dividing cells (58) and (especially after engineering) good resistance to gene silencing (59). Given this combination of features and the extensive work that has been undertaken in producing safe efficient lentiviral vectors, these are currently the preferred type of vector for long-term transduction of HSCs.

Summary: The advances made in the field of gene therapy are often directly applicable to humanized mouse studies and the growing consensus is that integrating viral vectors based on onco-retroviruses pose significant risks, self-inactivating lentiviral vectors based on HIV are the best available technology from the standpoint of safety and utility.

Promoters

In addition to the selection of an appropriate viral vector the choice of promoter is key to shaping a transduced HSC project. There are now a broad range of promoters which have been

characterized in sufficient detail to identify compact functional regions small enough to fit into a lentiviral vector (space is limited as the genome is not efficiently packaged once it gets much above 10kb in length). We briefly review the various promoters available highlighting good promoter choices for different applications. While there are many lentiviral vectors, we focus on those that have been tested to date in humanized mice following HSC transduction.

Constitutive promoters: Constitutive promoters are the most widely used in lentiviral vectors. However, for HSC transduction they have two important limitations – firstly all lineages of cells derived from the HSCs will express the transduced gene and secondly expression of extra genes in HSCs can often result in loss of self renewal and thus loss of the transduced HSC engraftment over time. Table 2 lists broadly available promoters that have been tested in HSCs in humanized mice. These promoters are best used for projects where several lineages of cells need to be transduced or where events early in hematopoiesis are under consideration.

T cell-specific promoters: Several T cell specific promoters have been indentified over the years and used in transfection studies and to create transgenic mice. The best characterized is the CD4 proximal promoter with or without the proximal repressor, which supports pan T cell or CD4 restricted gene expression, though there is some leakage in other lineages (60).

B cell-specific promoters: Several B cell specific promoters have been developed, however the best characterized of these in humanized mice are from the Tonelle group – who developed a combined CD19 promoter and Eμ enhancer (61, 62). The Rawlin's laboratory has described lentiviral vectors that also use the Eμ enhancer with either CD19 or Igβ (B29) promoters (63). While the B29 promoter seems to mediate stronger expression of GFP than the CD19 promoter both are reported to be leaky in the Rawlin's vectors. The Tonelle vectors on the other hand shows very low leakage and a much lower level of expression in cells of other lineages (62). This makes it the first choice for B cell specific expression to date.

Myeloid cell-specific promoter: Myeloid specific promoters that have been used to date in humanized mice have targeted specific subsets of myeloid cells. An HLA-DR specific promoter has been characterized in NOD SCID mice as a way of transducing human antigen presenting cells in humanized mice (64). Platelet specific promoter integrin αIIb has been shown to be active in human $CD34^+$ derived megakaryocytes and specifically expressed in mouse megakaryocytes and platelets following transduction of mouse HSCs (65, 66). The β-globin promoter has been described in similar detail as a potential gene therapy for sickle cell anemia and β-thalassemia, where progenitor cells were allowed to develop in mouse xenografts and used to repair a 'humanized' mouse genetic model of sickle-cell anemia (67, 68). While other promoters have been described they have not been used in humanized mice.

In conjunction with HSC culture, transduction of HSCs allows a powerful tool to be deployed to study the molecular mechanisms underlying human immunity and disease. While relatively few promoters have been extensively characterized in human HSCs in mice there are reasonably characterized promoters available to study many of the major lineages of cells as detailed in

Table 2. This is an approach that has not been extensively employed in humanized mice and represents an excellent opportunity to explore human biology.

Table 2: Summary of lentiviral vector promoters used in humanized mice

Promoter	Expression	Tested in humice	Notes	Ref
UCOE	Ubiquitous	No	Also promotes chromatin opening and inhibits gene silencing	(69)
UBC	Ubiquitous	Yes	Constitutive long term expression in NOD SCID mice	(70)
WAS	Ubiquitous	Yes	Constitutive long term expression in NOD SCID mice	(70)
PGK	Ubiquitous	Yes		(60)
CD4 proximal	Pan T/CD4	Yes	Expression depends on presence of CD8 repressor elements – some leakage in NK and myeloid cells	(60)
Eμ/CD19	B Cell	Yes	Low leakage strong specific expression	(61, 62)
B29	B Cell	Yes	Higher leakage than Eμ/CD19	(63)
HLA-DR	APC	Yes	Expression tested in NOD SCID mice	(64)
β globin	RBC	No	Tests carried out in mouse HSCs and human RBC progenitors allowed to develop in humanized mice	(67, 68)
Integrin αIIb	Platelet	No	Expression specificity demonstrated in mouse post HSC transduction not in human to date	(65, 66)
CD68	Macrophage	No	Expression specificity has been established in human cells for the 150bp proximal promoter and tested lentivirally *in vivo* in mice.	(71, 72)

Cytokine Administration in Reconstituted Animals

Once humanized mice have been generated experimentation can begin. For many studies it is desirable to further manipulate the system either by immunization/pathogen infection or by direct molecular stimulation. One of the easiest ways to do this is to alter the cytokine and/or growth factor environment to promote expansion and/or activation of particular subsets of human cells.

Indeed many of the new transgenic models discussed earlier were based on the results of cytokine administrations that demonstrably improved engraftment. As a result this tool is now being increasingly used to test hypotheses by perturbing existing models as well as to screen for factors that enhance humanization. This is achieved either by directly injecting human soluble factors or by hydrodynamic DNA injection, which induces expression of plasmid-encoded cytokines by the mouse liver. We briefly discuss the range of soluble factors

administered to date and the phenotypes they induce, summarizing all the collected data in Table 3.

Injection of soluble factors

In the early stages of humanized mouse development SCID mice were injected with various growth factors to enhance engraftment. The extremely low peripheral reconstitution level of the time (0.7% on average) (73, 74) was improved by injection of human IL-3 (increasing engraftment to ~4%) (75, 76). IL-2, IL-7, Flt-3L and SCF were shown to have no activity individually.

Co-treatment with erythropoietin (EPO), SCF, and/or GM-CSF/IL-3 fusion protein in adult human BM-transplanted SCID mice enhanced the proportion of human cells from <1% to 10-15% in treated animals, with development of multiple hematological cell lineages (77). However, treatment with human IL-3, GM-CSF and mast cell growth factor (MGF) had no effect on SCID mice engrafted with CB cells where higher levels (~15%) of engraftment could be achieved without treatment (78). This suggested that the CB cells respond differently to the murine microenvironment and/or GM-CSF and IL-3, possibly secreting key factors in a paracrine fashion. Based on the observations of improved engraftment after infusion of cytokines (75-77) and the relative bias toward B cell differentiation in several SCID mouse models, it has been confirmed that murine GM-CSF, M-CSF, IL-3, and TPO are not cross-reactive with human cells, whereas Flt-3L, IL-7 (which support lymphoid differentiation), and SCF are cross-reactive, at least to some extent.

With more permissive mouse models such as NOD/SCID, high levels of human cell engraftment were seen (11%-81% human $CD45^+$ cells in BM) (79). However, this tended to mask any effect of injected human cytokines such as IL-3, GM-CSF and EPO which showed little effect on hematopoiesis in this model (80). Only in less permissive strains such as C57BL/6 $Rag2^{-/-}$ $\gamma c^{-/-}$ mice where CB engraftment resulted in <1% human $CD45^+$ cells in the bone marrow could any effect be seen. Treatment of these mice with a mixture of IL-3, GM-CSF, and EPO showed limited improvement of human engraftment to ~18% human $CD45^+$ cells in the bone marrow (80). These observations demonstrate an important point – it is only in situations of limited engraftment that the beneficial effects of human cytokines are generally seen.

With the subsequent development of NOG and NSG mice, robust human cell engraftment is no longer a major problem. However, humanized mouse models still required further improvement For the following two reasons. First, the reconstitution of human NK cells and myeloid cells is generally poor (6, 81). Second, despite the capacity to support human B and T cell development from transplanted human HSCs, humanized mice have shown poor adaptive responses (7, 82, 83). Researchers thus focused on the improvement of reconstitution and function of specific human cell populations in humanized mouse models. Human NK cell reconstitution was intrinsically low in humanized mouse models because of the poor cross-reactivity of mouse IL-15. Kalberer *et al.* showed that a 1-week-long persistent *in vivo* treatment with various combinations of IL-15, Flt-3L, SCF, IL-2, IL-12 and megakaryocyte growth and differentiation factor (MGDF) could improve human NK cell development (from ~0.3% to ~5% in the spleen and bone marrow within the human $CD45^+$ cell population) in NOD/SCID mice injected with CB

$CD34^+$ cells (81). Similarly, in BALB/c $Rag2^{-/-}\gamma c^{-/-}$ mice engrafted with fetal liver HSCs, the frequencies of human $CD56^+$ $NKp46^+$ NK cells in all the lymphoid organs were very low (from 0.3% to 1.5% within human $CD45^+$ cell population) (84). Administration of a hIL-15/IL-15Rα complex increased NK cell numbers by 1.5 to 3 fold in all the lymphoid organs (84). This NK cell induction has been proposed as a means to assess antibody dependent cellular cytotoxicity in NSG mice reconstituted with CB $CD34^+$ cells (85). Most recently, Pek et al. also confirmed that administration of recombinant human IL-15 or an adenoviral vector expressing IL-15 are able to significantly enhance human NK cell development and maturation, particularly in the bone marrow and liver (86).

Limited *de novo* human thymopoiesis and peripheral T cell accumulation is a common feature of several humanized mouse models (4, 84, 87). Senpuku *et al.* showed that IL-18 treatment could stimulate the engraftment and migration of human $CD4^+$ and $CD8^+$ T cells in NOD/SCID mice transplanted with human peripheral blood lymphoid cells (88). Shultz *et al.* showed that administration of human Fc-IL-7 fusion protein results in dramatic increases in human $CD4^+CD8^+$ thymocytes and peripheral blood and splenic human T cells in NSG mice transplanted with mobilized adult $CD34^+$ cells (4). Similarly, it was demonstrated that treatment of human recombinant IL-7 showed transient effects on human thymopoiesis and plasmacytoid dendritic cells (pDC) development in young humanized $Rag2^{-/-}\gamma c^{-/-}$ mice (<10 week-old) engrafted with $CD34^+$ cells purified from human fetal livers without affecting peripheral T cell homeostasis (87). Recently, Huntington *et al.* reported that administration of human IL-15 boosts the numbers of both $CD4^+$ and $CD8^+$ T cells in the periphery and enhances B cell responses by increasing the frequency of antigen-specific responses following immunization (89).

Human myeloid and erythroid cells have also been induced in mice. When stromal cells secreting human EPO and IL-3 were co-transplanted into bone marrow with $CD34^+$ human HSCs in beige/nude/XID (bnx) mice, detectable levels of human red blood cells were transiently produced with an increase in hematocrit from 40% to 80% (75). A recent publication also confirmed that EPO and IL-3 treatment could further improve the peripheral human RBCs levels in humanized NOD/SCID and NOD/SCID/$\gamma c^{-/-}$ mice (90). These results show that GM-CSF, M-CSF and IL-3 are critical for human myeloid cell development in mice due to their high species-specific activities, but EPO shows cross-reactivity as mouse red blood cells also expand.

Expression of Human Cytokines by Injecting DNA Plasmids

Single injections of recombinant human cytokines in mice only produce a transient effect (typically 2 to 3 days). Repeated injections of large amounts of human cytokines are costly and labor intensive. To overcome these difficulties we developed hydrodynamic-tail-vein injection of DNA plasmids encoding cytokines in CB or fetal liver $CD34^+$ cell-engrafted NSG mice (6). This simple, inexpensive and efficient method allows the expression of flexible combinations of human cytokines *in vivo* for a long period (2-3 weeks for most of cytokines) following a single injection. We have tested several cytokines and combinations as detailed below.

We showed that the combination of IL-15 and Flt-3L expression dramatically improves the reconstitution and functions of human NK cells in both hematopoietic and non-hematopoietic tissues. NK cell frequencies were increased 3 to 10 fold depending on the organ examined, and absolute numbers of NK cells per tissue also increased 5 to 10 fold. These cells were functional showing enhanced responses to *in vivo* virus challenge (6).

After injecting plasmids expressing human GM-CSF, IL-4 and Flt-3L, we detected human $CD209^+$ DCs in blood, spleen, bone marrow, lung and livers. These DCs were hardly detected in untreated humice, yet they represented ~5% of human leukocytes after hydrodynamic injection. When an M-CSF plasmid was injected, the proportion of human macrophages increased by a factor of 10 to 20 in the tissues. These cytokine induced macrophages are functional because only the M-CSF treated humanized mice could exhibit significant *in vivo* macrophage responses when challenged by H1N1 flu or BCG (Tuberculosis) vaccine compared to non-treated humanized mice (unpublished data). Finally, when a combination of EPO and IL-3 plasmids were injected, human red blood cells (RBCs) were significantly induced in the peripheral blood, from below detection to a level of 1-4% in total RBCs (6). These results demonstrate that hydrodynamic injection offers opportunities for rapid testing of human cytokines and other genetically-encoded soluble molecules in humanized mice.

Summary

There are a significant number of human cytokines and growth factors that have been induced in humice (summarized in Table 3). To date the majority have caused expansion of cell subsets with the best results being seen when their target (whether whole graft or a particular subset) is poorly reconstituted in the starting model. Going forward a major focus of these sorts of short-term induction/injection studies will be modulating the function of specific human cell types.

Hydrodynamic-tail-vein-injection based cytokine expression shows certain advantages: 1) relatively long expression time courses (2-3 weeks per injection), 2) the expression level of each of several cytokines can be controlled by adjusting the dose of corresponding DNA plasmids used, and 3) low cost. This makes the strategy extremely useful for the rapid assessment of the systemic effects of particular soluble factors in humice.

Future Directions

This review has focused on research improving humanized mouse models, notably by creating new mouse strains and techniques to manipulate the human graft either directly (lentiviral infection) or indirectly (treatment with human soluble proteins). In addition a great deal of work has been done generating and characterizing infectious disease models in humice and creating models with additional human tissues, notably liver and thymus.

Going forward, three areas will likely attract significant effort:

Firstly, using the new tools available in conjunction with human infections which have until now lacked a small animal model. This will lead to new biological insights and allow the assessment of potential new therapies. The HIV research community, which has already produced over 100

research papers using humanized mice is leading by example. In this and other disease areas such as dengue, malaria and EBV infection all the tools are in place to test hypotheses about cell intrinsic factors (by using lentiviral vectors) and environmental effects of cytokines and growth factors by using hydrodynamic injection, new mouse strains and injection of recombinant proteins.

Secondly, the new technologies detailed in this review will allow an unprecedented chance to determine the genetic and environmental causes of autoimmune disease and hematologic cancer. By perturbing gene expression with lentiviral vectors and altering the 'normal' environment by introducing new combinations of human cytokines it will be possible to generate new disease models and in the process learn which factors are critical drivers of disease for human cells *in vivo*. This should serve as a powerful counterpoint to the sophisticated purely murine models currently in use as well as providing a timely platform to test biologics *in vivo* ahead of clinical trials.

Finally, the improvements seen in the last few years will be refined and integrated to make vastly better humanized mouse models. Already mouse strains with multiple knock-ins, knock-outs and transgenes are being characterized and the race is on to develop a mouse which combines all the advances in the myeloid and lymphoid lineages, reconstitution and cell function. As mice with fully functional human T, B, NK, macrophage and dendritic cells become available the focus will shift to generating mice with functional human neutrophils as well as the rarer granulocyte subsets and NK T cells.

In conclusion the work of the last five years has set the scene for what should be an explosion of biological insights and an increasingly prominent place in pre-clinical trials for humanized mice as they finally come of age over the next few years.

Table 3. Effect of human cytokines on human cells in various strains of humanized mice

Cytokine treatment	Method	Notes	Ref
IL-3	Injection	Enhances engraftment	(75, 76)
EPO,SCF,GM-CSF/IL-3	Injection	Enhances engraftment	(77)
IL-3,GM-CSF,EPO	Injection	Enhanced engraftment in the bone marrow	(78, 80)
IL-15,FL,SCF,IL-2,IL-12,MGDF	Injection	Enhances NK cell engraftment	(81)
hIL15/IL-15Rα	Injection	Enhances NK cells and T Cells	(84)
IL-18	Injection	Enhances direct T cell engraftment	(88)
Fc-IL-7	Injection	Enhances thymopoiesis and T cell engraftment	(4)
IL-7	Injection	Enhances thymopoiesis and pDC development	(87)
EPO, IL-3	Injection/ Hdam injection	Stimulates human RBC production	(6, 75, 90)
IL-2/IL-15 fusion +Flt-3l	Hdam injection	Improved NK cell reconstitution	(6)

GM-CSF + IL4	Hdam injection	Improved DC reconstitution	(6)
MCSF	Hdam injection	Improves monocyte/macrophage reconstitution	(6)
SCF,GM-CSF,IL-3	Transgene	Some myeloid improvement and more Tregs	(8)
TPO	Knock-in	Improves engraftment and enhanced myeloid cells in the bone marrow	(9)
GM-CSF, IL-3	Knock-in	Improved myeloid reconstitution esp. alveolar macrophages	(10)
M-CSF	Knock-in	Improves monocytes/macrophage reconstitution in all organs.	(11)

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