Lymphoid development; a dynamic interplay of timing and dosing
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New prospective tools to study human hematopoiesis

Mice and Men
Most of the currently available knowledge on hematopoiesis is derived from studies performed in mice. Rodents have been praised as prime experimental subjects, because they quickly reach sexual maturity and are easily kept and bred in captivity. More importantly, mice exhibit a high degree of genetic homology with humans, and reports on the sequencing of human and mouse genomes revealed that only approximately 300 genes are unique to one species or the other, in the context of over 20,000 human genes described to date (1). Therefore, mice are the mainstay of in vivo immunological experimentation, and in many respects they mirror human biology remarkably well. Nevertheless, next to the abundant parallels, differences between mouse and human immunology exist (2). There has been a disposition to ignore differences and in many cases, perhaps, make the assumption that what is true in mice can be directly translated to men. By making such assumptions we run the risk of overlooking aspects of human immunology that do not occur, or cannot be modeled, in mice. Clearly, with so many paradigms that translate well between both species, and with the relative ease with which mice can be genetically manipulated, mouse models will continue to provide important information for many years to come. However, especially when considering future therapeutic options for human individuals, we should be cautious and treat mouse data more as a lead to expected findings in the human setting.

Pathogens displaying unique human tropism underscore the differences in human and mouse constitution. This tropism is exhibited by various important infectious agents, causing diseases which continue to heavily burden our global society. Chronic viral infections, including those caused by hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) currently afflict more than 500 million people worldwide, cumulatively resulting in more than 3.5 million deaths per year (3, 4). Bacterial and parasitic diseases have a similarly high impact; Mycobacterium tuberculosis frequently establishes persistent infections, with an estimated 2 billion carriers around the globe and an annual mortality of close to 1.7 million individuals (5). Endemic and epidemic malaria results in severe disease in an estimated half a billion people each year, and causes over 1.5 million deaths annually (6-9). Many infectious diseases disproportionately affect marginalized populations in the poorest countries, exacerbating disparities in economic growth and creating downward spirals of poverty (6-9). Although progress has been made in the prevention and treatment of these infections, more effective, tolerable and affordable therapies are urgently needed.
The development of novel intervention strategies has been hampered by the lack of robust, cost effective and most importantly predictive animal models. While rodents and non-human primates have been employed in biomedical research as well as in drug and vaccine development, they often do not yield reliable pre-clinical results that translate into effective human treatments. For decades large primates, especially chimpanzees, have been used to study immunobiology and candidate therapies against human pathogens such as HIV, HBV (10) and HCV (11). Although humans and chimpanzees are genomically greater than 98% identical, these small differences can significantly influence disease pattern and outcome (12). For example, chimpanzees and humans do not share any human leukocyte antigen (HLA) class I alleles, the common human allele (HLA-A2) is completely absent in chimpanzees, and clear differences in the MHC class II region are also observed. As a consequence non-human primates often do not completely reproduce the pathophysiology of human diseases. In addition, use of these large primates is expensive, banned in many countries due to ethical concerns, and experiments with limited numbers of outbred animals are plagued by considerable inter-individual variability and consequently limited reproducibility. These deficiencies exacerbate the considerable roadblocks to vaccine development. Current regulations for human vaccine testing – among others the stringent requirement for good manufacturing practice (GMP) grade material for phase I studies – have led to an explosive increase in clinical development costs. To bridge the translational gap, novel strategies are needed for rapid and cost-efficient screening, selection, and prioritization of the most promising candidates. None of the existing animal models adequately addresses the needs of vaccine developers, nor do they provide the detailed understanding of the human immune response that is required for generation of vaccine strategies against pathogens that have closely co-evolved with humans. Investigation of human biology in vivo is hampered by severe ethical and practical limitations – including use of placebos and experimental drugs prone to resistance selection, the risk of discrimination or stigmatization as a consequence of having participated in the research, limited sample procurement, and heterogeneity of the study population. These limitations and lack of suitable animal surrogates create a seemingly insurmountable barrier to conducting essential pre-clinical investigations.

**Humanized mice: modeling human immunology and infectious disease for basic biology and preclinical applications.**

“Humanized” mice have recently emerged as powerful tools in the investigation of human diseases (reviewed in (13-16)). These are amenable small animal models transplanted with human cells or tissues, or equipped with human transgenes, and may be ideally suited for
direct investigation of human infectious agents. Successful engraftment depends on avoiding rejection and maximizing tissue function, ensured by correct localization and appropriate tissue support by host factors. Despite the challenges, humanized mouse technology has made rapid progress over the last few years and it is now possible to achieve high levels of human chimerism in various compartments, particularly the immune system, liver and muscle. State-of-the-art mice reconstituted with a human immune system (HIS mice) principally recapitulate the development of hemato-lymphoid compartments (17-19). This offers a basis for two lines of research: (a) it provides the first and unique opportunity to study and manipulate in vivo human hematopoietic development at all stages and all sites of differentiation, and (b) it allows for the examination of human tropic pathogenic mechanisms as well as potentials for their treatment.

**Human immune system (HIS) mice as an immunological tool**

To assess the role of specific genes in human lineage commitment and differentiation, genetic manipulation of the human HSC before generation of HIS mice represent an attractive approach. To confirm observations generated in vitro with human immune cells in an in vivo setting, Schotte et al. elegantly demonstrated an exclusive block in pDC development when the transcription factor Spi-B was knocked down by SpiB-RNAi in the injected HSC (20). Alternatively, the potentials of specific human cytokines can be further investigated. For a long time, the exact role of IL-15 in human NK differentiation and/or maintenance has remained not fully elucidated. In the mouse, IL-15 is reported to maintain the viability and support the proliferation of NK cells, rather than acting as a differentiation factor, because NK cell precursors are present in normal numbers in mice deficient for IL-15 or its receptor components (21). It is not known whether NK precursors are present in γc-deficient human individuals, partly because our knowledge of early NK cell precursors in humans is limited, but mostly due to the limited availability of the patient's tissues combined with the paucity of NK precursors (reviewed in (22)). A recent report by Huntington et al. finally sheds light on the role of human IL-15 in an in vivo context (23). In this study, HIS mice were inoculated with IL-15/IL-15Rα complexes, which resulted in the induction of extensive NK cell proliferation and differentiation, and accumulation of CD16+KIR+ NK cells. This was shown not to uniquely depend on enhanced survival or preferential responsiveness of this subset to IL-15, suggesting an intimate relationship between IL-15 and NK cell differentiation and homeostasis. Additionally, over-expression of TSLP in transplanted human HSC generated the first in vivo proof of the effect of TSLP on human T cell development and CD4+CD25hi generation (see chapter 6). Previously, evaluation of
cytokine function was limited to ex vivo and in vitro assays, which do not necessarily reflect the real impact in physiological conditions.

Taken together, the HIS mouse models offer the first opportunity to investigate the effects of genes and cytokines on the differentiation and the maintenance of human leukocytes, in an in vivo, multi-lineage context. Nevertheless, we have to keep in mind that the HIS mouse immune system is not identical to the immune system of actual human beings. It remains an approximation, in the context of a prospective experimental system. In that light, experimental data generated in this model, shed light on the potential effects of introduced factors on the human (developing) immune system. The effects observed may be not be identical in the context of a complete human immune system in a human individual.

**Current status and future prospects of mice carrying the target tissues of human pathogens**

HIS mice are already showing potential as the only available small animal challenge model for HIV infection, a valuable platform for testing the efficacy of anti-viral compounds. Importantly, mice can be infected with HIV not only intravenously but also by relevant intravaginal and intrarectal routes, making this model particularly attractive for pre-clinical validation of anti-microbial agents that are active at mucosal surfaces (24-26). Recent disappointing clinical trial outcomes with anti-HIV microbicides could have possibly been predicted and avoided if sufficient pre-clinical data of this type had been available (26).

Despite their promise, immune responses to infection remain suboptimal in HIS mice. For instance, tetanus toxoid immunizations result in lower antibody levels in HIS mice than those achieved in human adults (19). Only very few HIV-infected HIS mice develop virus-specific antibodies, and thus far HIV-specific T cell responses have not been observed (27). Epstein Barr virus (EBV) infection of HIS mice results in activation of T and B cells, but can progress to B cell lymphoma, indicating that these mice cannot control the infection (18, 19, 28). Similarly, experimental immunization with the approved human HBV vaccine leads to antigen-specific B cell responses in only 40-50% of HIS mice (P. Becker, N. Legrand, C. van Geelen et al., manuscript in preparation). Taken together, these data suggest that immunity in humanized mice is not yet comparable to humans, and further development is required before these mice can be employed in screening human vaccine candidates.
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Generation of a robust predictive model for human immune responses requires significant improvement in HIS mouse reconstitution and function. The overall cellularity of human cells in HIS mice is below the desired levels (17, 19). Likewise, IgM and IgG serum titers are substantially lower in HIS mice as compared to humans (29). Human HSC are insufficiently maintained after xenograft transplantation, and differentiation into particular lineages, such as erythro-myeloid cells, is particularly ineffective. Last, the inadequate formation of higher order lymphoid structures may be central to the limited immune responses observed in HIS mice. Several strategies to improve human hematopoietic reconstitution in HIS mice have been reviewed recently (13, 15), including enhancing the ablation of endogenous mouse subsets to create ‘space’ for human cells, counteracting active graft destruction, and expressing human MHC molecules to ensure proper T cell education and homeostasis. Additionally, as discussed in chapter 4, the administration of exogenous cytokines to overcome impaired biological cross-reactivity between mouse and human are examined. Although each of these modifications may only incrementally improve the functionality of HIS mice, the additive (and potentially synergistic) effects of these different improvements create optimism that a predictive model for the human immune response in mice can be achieved in the near future. Use of these advanced animal models for vaccine testing is expected to enhance our ability to predict performance in humans, and will thereby enable rapid and efficient selection of the best vaccine candidates for translation into the clinical development pipeline.

‘Multi-tissue’ humanized mouse models

Mice with humanized immune systems already represent the model of choice for various lymphotrophic pathogens, including HIV, EBV, and Kaposi’s sarcoma-associated herpes virus (KSHV) (30). The addition of human hepatic tissue holds promise for the study of hepatotropic pathogens, such as HBV (31, 32), HCV (31, 33), dengue virus (34), yellow fever virus, and malaria (35, 36), all of which previously lacked accurate and amenable in vivo models. Other areas of investigation, such as autoimmune processes and human tumorigenesis, are also benefiting from humanized mouse technology (14, 16). Mouse models that combine several humanized compartments will increase the specific questions that can be addressed, and a collection of humanized mice “on demand” could be envisaged. For example, mice with a human immune system and supplementary human lung tissue could model tuberculosis or cytomegalovirus (CMV) pathogenesis. Furthermore, preclinical evaluation of vaccine potency could be performed in mice engrafted with tissues efficiently supporting various pathologies, and safety, efficacy, and pharmacokinetics studies
of drugs and therapeutic biologicals (37) could be addressed in a tissue- and cell subset-specific manner.

**Genetic tools**

To address fundamental questions about the role of genes and cytokines in lineage decisions, commitment and cell maintenance in chapters 4, 6, 7 and 8, we made use of viral systems to affect gene expression. Both lentiviral, as well as retroviral, vector-mediated transduction of stem cells was used to enforce increased expression of genes of interest. Such gene delivery approaches ensure stable gene integration into the host cell genome and maintenance of persistent gene expression.

The choice of the promoter which is driving the expression of the gene of interest in the lentiviral vectors, appeared of the upmost importance. Initial attempts to assess altered gene function during human hematopoiesis in HIS (BALB-Rag/γ) mice delivered disappointing results. With a CMV promoter driving gene expression and a human elongation factor (EF)1α promoter driving marker expression separately, we found high recovery of marker-positive cells but no over-expression of the gene of interest. There is strong evidence suggesting that CMV promoter-driven expression in vivo is often declining over time (38) and it cannot be excluded that CMV promoter silencing occurs in the human cells transduced with our lentiviral vectors e.g. through DNA methylation and histone deacetylation (39, 40). Therefore we set up comparative experiments with lentiviral vectors containing different promoters to drive expression of the enhanced green fluorescent protein (EGFP), and compared marker expression over time in transduced HSC, which were transplanted into HIS mice. Comparison of CMV, the phosphoglycerate kinase (PGK) and the human EF1α promoters demonstrated that EF1α was best capable of inducing high marker gene expression, both at early (before injection and after 5 weeks) and late (12 weeks) time points of analysis. Subsequently, all lentiviral transduction experiments were performed with vectors containing the EF1α promoter to drive expression of our genes of interest, upstream of an internal ribosome entry site (IRES) and a marker gene such as EGFP.

Virus-mediated gene transduction systems are attractive techniques to genetically influence human T cell development. Murine immunology can extensively be analyzed with (conditional) knock-out and knock-in systems, the latter having as major advantage of close to physiological levels of gene expression. These strategies are unfortunately inaccessible for human immunological studies. However, virus-mediated gene transfer technology offer
specific advantages of their own, such as the possibility to introduce genes before or after defined checkpoints of cellular development.

Currently, lentiviral vectors are promising tools for the development of gene therapy. With their additional abilities; (a) to transduce non-dividing cells, (b) their safer integration pattern into the host’s genome (further away from the 5’ end of endogenous genes) and (c) their genomic stability, allowing accommodation of large genomic sequences, lentiviral vectors are overtaking the field of gamma-retroviral vectors (41). Several lentiviral vectors have been or are currently being used to offset advanced forms of HIV infection, treat inherited disorders acting upon hematopoietic cells, or transduce neuronal cells of the central nervous system for the treatment of Parkinson disease (reviewed in (42)). These offer promising effective therapeutic means for patients in the short and long-term future.

**T lineage fate decisions**

The observations made in chapter 8 highlight the plasticity that defines early T cell precursors. During many cell divisions and generations, they stay uncommitted to the T cell fate and maintain alternative potentials as different as monocyte or dendritic cell fates (reviewed in (43)). This prolonged plasticity of developing T cells is maintained throughout development up to, or close to, the point that TCR-mediated expansion is dominating. It is suggested to be debit to two major groups of factors: (a) the stem- and progenitor-associated regulatory genes, like PU.1, which display only gradual silencing over time; and (b) the promiscuous regulatory factors required for T lineage commitment themselves, like Id2, SCL, C/EBPα and GATA2 (44) which paradoxically also maintain non-T cell developmental options. We observed that dual over-expression of the Id2 and the TCRαβ genes preserved the bipotential NK/T cell fate and the plasticity characteristic of normal early T cell development.

In comparison, developing B cells harbor an intrinsic, cell-autonomous fidelity mechanism from a relatively early stage. The B cell program is based on three transcription factors operating in a classic feed-forward regulatory cascade: EBF and Pax5 are B lineage specific (45) and function together with E2A as direct activators of most B lineage genes. Additionally, Pax5 itself is a major supporter of B lineage commitment through its direct interference with other lineage decision programs (46). Overall, the T cell-specification process is quite different in terms of regulatory mechanisms than B cell specification, as it is based on the
mobilization of a variety of factors that may not individually be specific for regulation of T cell genes. These factors induce a delayed commitment towards the T cell lineage, which is reflected in the prolonged periods of Notch triggering required to divert TCR x Id2 transduced thymus progenitors away from the NK cell lineage, even though a fully arranged TCRαβ complex was already expressed (see chapter 8). Notch-Delta signaling appears to be crucial throughout an extended period to buffer pro-T cells against activity of their “own” factors, to maintain a bias in favor of the T lineage outcome. Hence, the accelerated T cell commitment of PNT transduced with a full TCRαβ (see chapter 7) is only phenotypic, and prolonged periods of Notch triggering are probably required to generate a fully committed antigen-specific T cell pool.

Without simultaneous TCR over-expression, the activity of a bHLH transcription factor is necessary for T lineage differentiation of bipotential T/NK precursors. The presence of Id2 in thymic progenitors dictates NK cell development and as no other functions of Id2 except for blocking E protein activity are known, this can be interpreted as a kind of default pathway. Considering that extensive analysis of the molecular mechanisms that control TCRαβ and TCRγδ T lineage differentiation revealed that TCRαβ T cell development is far more complex than γδ T cell development, the γδ lineage may likewise be seen as a default T cell state (47). Accordingly, the mature DN T cells generated from progenitors transduced with TCRxId2 are potentially the product of phenotypic and functional ‘commitment’ to the T cell lineage induced by the introduced TCR and a developmental arrest induced by Id2. Overall, this situation is imposing the TCRαβ lineage decision as a default pathway with Id2 additionally retaining plasticity of cell fate.

**In vitro generated T cells**

The adoptive transfer of cytotoxic T lymphocytes (CTL) is an attractive approach to supply cancer- or pathogen-reactive T cell responses that are otherwise either absent or insufficiently present in patients. The therapeutic efficacy of this strategy has been demonstrated in clinical trials for the treatment of chronic myelogenous leukemia, cytomegalovirus-mediated disease, and Epstein-Barr virus-positive B cell lymphomas (48-50). Its widespread use is however limited by the complexity in obtaining suitable T cell clones and generating sufficient amounts of CTL in vitro, especially for the treatment of solid tumors (51). A new opportunity for antigen-specific T cell therapy is offered by TCR gene transfer, which consists of the introduction of cloned TCR genes, with known antigen specificity, into T lymphocytes.
Recent T cell-based therapeutic approaches, including two clinical trials, successfully applied genetic engineering of T cell specificity by TCR gene transfer. The first trial in humans demonstrated that TCR gene-modified autologous T cells persisted for over 2 months and reduced tumor burden in two out of fifteen melanoma patients (52). This was clearly less than that of previous clinical trials by the same group which involved infusion of ex vivo expanded tumor-infiltrating lymphocytes (53, 54). In this clinical trial, the frequency of T cells with strong expression of the introduced TCR was rather low and variable. Nonetheless, these results illustrate that TCR gene transfer is feasible in a clinical setting, although optimization is required. The other clinical trial used an allogeneic CTL cell line that was injected directly into the tumor (55). The clinical outcome was disappointing with only one partial response among 15 treated patients. The precise reason for the limited responsiveness in both studies remains unclear, although several, non-exclusive, involved mechanisms have been put forward. First of all, the characteristics of the T cells used for adoptive T cell therapy appear of major influence. Our knowledge on the optimal differentiation state and effector functions that infused T cells should exhibit is still incomplete, but it appears that strategies that avoid the development of terminally differentiated T cells during in vitro culture deliver T cell populations with superior function in vivo (56-58). Additionally, administration of T cells under conditions that permit homeostatic expansion of the infused cells creates a desirable setting for adoptive T cell therapy of unmodified (59) and TCR gene-modified T cell populations (60).

Secondly, with the transfer of both a single TCR α-chain (TCRα) and β-chain (TCRβ) to generate the TCR of interest, it cannot be excluded that pairing with endogenous TCRα and TCRβ occurs (61), generating additional surface TCRαβ complexes consisting of mixed dimers. Simultaneous expression of mixed dimers and the TCR of interest leads to cell surface dilution of the antigen-specific TCR. Moreover, as the specificity of the mixed TCR dimers cannot be predicted, the formation of unwanted auto-reactivity is a risk factor (62). One strategy to address this pitfall is TCRαβ gene transfer into γδ lineage cells, since δ and γ TCR chains cannot form heterodimers with α and β TCR chains (63). Redirection of γδ T cells therefore allows TCRαβ gene transfer without formation of mixed TCR dimmers (64). However, another safety issue in TCR gene transfer still abides: the chance of robust activation of previously anergic self-reactive γδ T cells via their new TCRαβ.

Alternatively, an attractive approach to overcome the hybrid TCR formation is TCRα gene
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transfer into human hematopoietic progenitors, which do not express alternative TCR chains yet, followed by in vitro T-cell-lineage differentiation (described in chapter 7). With this approach naïve T cells are generated and problems linked to the use of terminally differentiated T cells are circumvented. Additionally, enforced expression of transduced TCR chains by differentiating HSC facilitates the repression of Rag genes expression, such that endogenous TCRβ are not expressed (65) and no alternative β chains are detected on the cell surface (chapter 7). However, full TCRα allelic exclusion was not proven and most likely this is incomplete. Further technical development to optimize preferential pairing of the introduced chains, such as the manipulation of the transmembrane-association domains of TCRα and TCRβ (66), will help to reduce further the risk of TCR mis-pairing.

Notch-based culture systems, such as mouse OP9 stromal cells expressing DL1 Notch-ligand, are demonstrated to be applicable to generate T cells from human cord blood but also human adult bone marrow-derived CD34+ progenitor cells (67). Recently, a stromal cell-free Notch-based culture system using immobilized DL1 was established which allowed 2-fold expansion of cord blood-derived CD34+ cells before xeno-transplantation to generate HIS mice (68). These data indicate that this technique can be converted to a clinically applicable therapeutic method. Furthermore, to enhance T cell reconstitution and graft versus tumor (GVT) activity after T cell-depleted allogeneic hematopoietic stem cell transplantation (HSCT), the integration of TCR gene transfer technology into the (immobilized-DL1 based) T cell precursor expansion protocols, represents a promising novel strategy (reviewed in (69)).

In conclusion, with the large amounts of studies aiming at (a) improving displacement of endogenous TCR from the cell surface by transferred TCR, (b) increasing TCR expression level, (c) identifying the best target antigens (reviewed in (70)), and (d) developing optimized lentivirus-based vectors for transduction with less effect on differentiation state (57) and decreased risk of insertional mutagenesis in progenitor cells (42), it is reasonable to believe that the TCR gene transfer technology is a promising therapeutic approach.
References


