Published in final edited form as:

Immunol Res. 2009 ; 44(1-3): 112–126. doi:10.1007/s12026-008-8088-z.

Suppression of HLA Expression by Lentivirus-mediated Gene Transfer of siRNA Cassettes and *In Vivo* Chemoselection to Enhance Hematopoietic Stem Cell Transplantation

Katrin Hacke^{*},

Department of Medicine, David Geffen School of Medicine, University of California Los Angeles (UCLA)

Rustom Falahati^{*},

Division of Hematology / Oncology, Department of Medicine, University of California San Francisco (UCSF)

Linda Flebbe-Rehwaldt,

Division of Hematology / Oncology, Department of Medicine, University of California San Francisco (UCSF)

Noriyuki Kasahara, and

Department of Medicine, David Geffen School of Medicine, University of California Los Angeles (UCLA)

Karin M. L. Gaensler^{**}

Division of Hematology / Oncology, Department of Medicine, University of California San Francisco (UCSF)

Abstract

Current approaches for hematopoietic stem cell (HSC) and organ transplantation are limited by donor and host-mediated immune responses to allo-antigens. Application of these therapies is limited by the toxicity of preparative and post-transplant immunosuppressive regimens and a shortage of appropriate HLA-matched donors. We have been exploring two complementary approaches for genetically modifying donor cells that achieve long-term suppression of cellular proteins that elicit host immune responses to mismatched donor antigens, and provide a selective advantage to genetically engineered donor cells after transplantation.

The first approach is based on recent advances that make feasible targeted down-regulation of HLA expression. Suppression of HLA expression could help to overcome limitations imposed by extensive HLA polymorphisms that restrict the availability of suitable donors. Accordingly, we have recently investigated whether knockdown of HLA by RNA interference (RNAi) enables allogeneic cells to evade immune recognition. For efficient and stable delivery of short hairpin-type RNAi constructs (shRNA), we employed lentivirus-based gene transfer vectors that integrate into genomic DNA, thereby permanently modifying transduced donor cells. Lentivirus-mediated delivery of shRNA targeting pan-Class I and allele-specific HLA achieved efficient and dose-dependent reduction in surface expression of HLA in human cells, and enhanced resistance to allo-reactive T lymphocyte-mediated cytotoxicity, while avoiding non-MHC restricted killing.

^{**}To whom correspondence should be addressed, at: Department of Medicine, Division of Hematology / Oncology, University of California San Francisco (UCSF), 533 Parnassus Ave., M-1286, San Francisco, CA, 94143, gaensler@medicine.ucsf.edu.

^{*}R. Falahati and K. Hacke contributed equally to this article

Complementary strategies for genetic engineering of HSC that would provide a selective advantage for transplanted donor cells and enable successful engraftment with less toxic preparative and immunosuppressive regimens would increase the numbers of individuals to whom HLA suppression therapy could be offered. Our second strategy is to provide a mechanism for *in vivo* selection of genetically modified HSC and other donor cells. We have uniquely combined transplantation during the neonatal period, when tolerance may be more readily achieved, with a positive selection strategy for *in vivo* amplification of drug-resistant donor HSC. This model system enables the evaluation of mechanisms of tolerance induction to neo-antigens, and allogeneic stem cells during immune ontogeny. HSC are transduced *ex vivo* by lentivirus-mediated gene transfer of P140K-O⁶-methylguanine-methyltransferase (MGMT^{P140K}). The MGMT^{P140K} DNA repair enzyme confers resistance to benzylguanine, an inhibitor of endogenous MGMT, and to chloroethylating agents such as BCNU. *In vivo* chemoselection enables enrichment of donor cells at the stem cell level. Using complementary approaches of *in vivo* chemoselection and RNAi-induced silencing of HLA expression may enable the generation of histocompatibility-enhanced, and eventually, perhaps “universally” compatible cellular grafts.

Keywords

HLA – Human Leukocyte Antigen; **MHC** – Major Histocompatibility Complex; **shRNA** - short hairpin-type RNAi; **RNAi** – RNA interference; **MGMT** - O⁶-methylguanine-DNA-methyltransferase; **MAG** - MGMT^{P140K}-2A-GFP lentivirus vector; **BU** - Busulfan; **BG** - Benzylguanine; **BCNU** - 1,3-bis(2-chloroethyl)-1-nitrosourea; Lentivirus

Introduction

Allogeneic transplantation: opportunities and limitations

Sibling or matched unrelated allogeneic transplants of adult or cord blood hematopoietic stem cells (HSC) remain the only curative therapy for patients with hereditary disorders including hemoglobinopathies, immune deficiency, and inborn errors of metabolism. However, the toxicity of myeloablative preparative regimens, risks of graft vs. host disease (GVHD), infectious complications of immunosuppression, and limitations in the availability of suitable related or unrelated donors limit the number of patients to whom allogeneic bone marrow (BM) transplants can be offered. Particularly in neonatal models, myeloablative transplantation approaches have been associated not only with higher morbidity and mortality, but also with subsequent abnormal development and growth (1).

Approaches to decrease toxicity associated with HSC transplantation include reducing the intensity of preparatory regimens and T cell depletion to decrease GVHD. Partially mismatched donors such as haploidentical parents and siblings have been used to increase the available donor pool. Indeed, the successful use of haplo-identical donors for the treatment of severe combined immunodeficiency disease, pioneered by Dr Good and other colleagues, was one of the landmark advances in the use of transplantation to treat this disorder. However, immune responses against donor-specific antigens and immune-mediated rejection of transplanted hematopoietic progenitor cells and resultant graft failure remain important obstacles. To date, the primary strategies for avoiding immunological rejection of allogeneic transplants have been to minimize antigenic differences between donor and recipient by matching Human Leukocyte Antigens (HLA; the Major Histocompatibility Complex (MHC) antigens in humans), and to condition the host by suppression/ablation of the host immune system using irradiation or potent chemotherapy.

Limitations for application of allogeneic transplantation: HLA matching

The most important loci for graft survival are the HLA Class I antigens A and B, and the Class II antigen DR (Figure 1). However, HLA are highly polymorphic with more than 220, 460, 110, and 360 molecularly defined epitopes for HLA-A, B, C, and DR respectively. Mismatching of the serological antigens increases the probability of graft failure (2-4). Even with serological cross-matching, small molecular genetic differences may result in graft rejection (5-7). Further, graft vs. host disease, associated with HLA antigen mismatching between donor and recipient, results in considerable morbidity and mortality (8,9). Despite the growing size of national and international registries there continues to be a lack of suitable donors for both stem cell and organ donation. Procuring suitable HLA-matched donors represents the major limitation to the field of organ and stem cell transplantation. Finally, even with well-matched HLA-A, B, and DR antigens, potent immunosuppressive regimens are required. Thus, for patients there is a fine balance between “too little” immunosuppression and graft rejection, or “too much” immunosuppression resulting in infection and toxicity to the recipient, and in some cases leading to post-transplant malignancies (10,11).

HLA down-regulation as a strategy for reducing donor/host immune responses

We have been pursuing a novel strategy for genetically modifying graft cells to achieve long-term suppression of cellular proteins that elicit or potentiate host immune responses against mismatched donor antigens. In principle, modifications to reduce or eliminate expression of specific HLA expression on donor cells would reduce alloreactive immune responses, the need for full HLA matching, and the need to suppress the host immune system. The goal of this approach is to achieve down-regulation of HLA expression sufficient to alleviate graft rejection without deleteriously impacting immune responses against foreign pathogens. Support for the concept that long-term suppression of HLA may enable donor cells to evade host immune responses comes from studies showing that grafts from MHC class I and class II “knockout” mice survive longer than controls (12,13). However, antibody-based strategies such as graft pretreatment with haplotype-specific anti-Ia mAb, haplotype-specific immunoconjugates, or mAb directed against graft antigen presenting cells (APC), adhesion or costimulation molecules, were found to have little efficacy in promoting acceptance of allografts in immunocompetent hosts (13).

Virus-based gene transfer vectors, such as lentivirus vectors, adeno-associated virus (AAV) vectors, and helper-dependent adenovirus vectors may be used to transduce graft cells with sequences encoding small interfering RNAs (siRNAs), anti-sense mRNA, and ribozymes directed against critical immune recognition molecules or immunostimulatory factors. Engineering the graft using these approaches could decrease donor cell immunogenicity and increase the pool of unrelated bone marrow donors, while reducing the need for toxic myeloablative pre-conditioning or immunosuppressive regimens.

Recent studies have demonstrated that adenoviral-mediated expression of an anti-human MHC I single-chain intrabody achieved ‘phenotypic knockout’ of Class I HLA in human primary keratinocytes and in endothelial (HUVEC) cells (14,15). Intrabody-transduced cells were protected from lysis by sensitized allogeneic cytotoxic T lymphocytes (CTLs), while control cells from the same donor were not (15), establishing proof-of-concept for targeted inhibition of MHC expression in reducing immunogenicity of adult tissue allografts. However, in terms of practical implementation, adenoviral-mediated approaches are unlikely to have long term benefits for graft survival as adenoviruses are a non-integrating vectors that direct short-lived expression and are highly immunogenic due to the leaky expression of viral proteins. Further, the synthetic anti-MHC intrabody protein itself also represents a neo-antigen that may be immunogenic.

Application of siRNA-based methods for knockdown of HLA expression

In contrast to adenoviral vectors, the use of oncoretrovirus- or lentivirus-based gene transfer vectors enables long-term transduction of donor HSC and expression of sequences encoding siRNAs, anti-sense mRNA, or ribozymes directed against immune recognition molecules such as HLA, or co-stimulatory molecules such as CD80. These vectors could also be used for gene delivery of specific ubiquitinating enzymes, dominant-negative inhibitory proteins, and transmembrane or secreted immunosuppressive factors. The effect of such manipulations will be to decrease immunogenicity of donor cells and associated host and graft mediated immune responses. A potential limitation of this approach is that complete silencing of HLA may render donor cells susceptible to recognition and attack by non-HLA-restricted effector cells such as natural killer (NK) and lymphokine-activated killer (LAK) cells. It may be necessary to modulate siRNA-mediated silencing and HLA expression to a level that does not allow recognition by alloreactive T cells and yet will not elicit NK-mediated lysis. Alternatively, precise knockdown of specific HLA alleles may be used to nullify potential mis-matches. For example, lentiviral-mediated class- and gene-specific silencing of HLA would broaden the application for transplantation of existing human embryonic stem cell (hESC) pools in future regenerative medicine approaches by reducing the problem of HLA mismatches in recipients of hESC-derived grafts (16,17).

RNA interference (RNAi) is a potent genetic tool for silencing gene expression that triggers post-transcriptional degradation of homologous transcripts through a multi-step mechanism involving double-stranded siRNA (18,19). Recently, Cooper, Rossi and colleagues demonstrated that T cells stably transfected with plasmids containing siRNA expression cassettes showed HLA down-regulation that provided protection from cytolysis even when the transfected cells were loaded with a target peptide and challenged with peptide-specific CTLs (20). However, these stable transformants expressing high copy numbers of siRNA could only be isolated by co-transfection and selection with antibiotic resistance genes in an immortalized Jurkat T cell line. This methodology is not feasible for hematopoietic progenitors and particularly quiescent stem cells. Thus, our focus has been to use lentivirus-based vectors, capable of highly efficient transduction of quiescent cells, including HSC. Permanent integration into the host cell genome, provides the potential for long-term suppression of HLA in adult cell and organ transplantation.

Advantages of Lentiviral-based approaches for HLA-silencing

In contrast to adenovirus, AAV, and non-viral gene transfer approaches, both oncoretroviral vectors and lentiviral vectors have the capacity to integrate into the host genome and direct stable gene expression. Onco-retroviral vectors remain one of the most frequently utilized gene delivery modalities in clinical trials to date. During the retroviral life cycle the reverse transcriptase converts the viral RNA genome to double-stranded DNA, which is then permanently integrated into the chromosomes of the host cell, enabling long-term gene expression. Since native coding sequences have been completely replaced by therapeutic genes of interest there is no possibility that expression of native viral genes might lead to immunological rejection of cells transduced by replication-defective retrovirus vectors. Progressive understanding of the characteristic limitations of MLV-based vectors has spurred numerous improvements in vector production and transduction methods, particularly for *ex vivo* applications, and has led to their more judicious application to relevant clinical scenarios. In this regard, arguably one of the leading successes to date has been the amelioration of X-linked severe combined immunodeficiency (SCID); however, enthusiasm regarding this success has been tempered by sobering reminders of the potential for risk, as well as benefit, of viral-mediated gene integration into host chromatin (21,22).

Among the most promising basic advances in gene delivery vector technologies in recent years has been the development of lentivirus-based vector systems. Lentiviruses are complex retroviruses that contain additional regulatory and pathogenicity-enhancing “accessory” genes in addition to the *gag*, *pol* and *env* structural proteins classically expressed by oncoretroviruses (Table 1). Although the overall life cycle of lentiviruses is similar to that of oncoretroviruses, there are several major differences between these vectors. Most notably, oncoretroviruses such as MLV can only transduce cells that divide shortly after infection, because the MLV pre-integration complex cannot achieve chromosomal integration in the absence of nuclear envelope breakdown during mitosis.

In contrast, lentiviruses can infect non-proliferating cells, owing to the karyophilic properties of the lentiviral pre-integration complex, which allows recognition by the cell nuclear import machinery (Table 1 and Figure 2). Correspondingly, lentiviral vectors can transduce cell lines that are growth-arrested in culture, as well as terminally differentiated primary cells including hematopoietic stem cells, neurons, hepatocytes, cardiomyocytes, endothelium, alveolar pneumocytes, keratinocytes and dendritic cells [18-26]. Hence, there is increasing interest in the development of vector systems based on a wide variety of lentiviruses, including human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). We have focused primarily on HIV-based lentiviral vectors for permanent integration of transgenes to achieve long-term modification of cellular phenotype, as this technology has progressed the most rapidly and is already in clinical trials.

Development of lentiviral vectors for silencing of HLA Class I expression

To test two different targets for HLA silencing, candidate siRNA sequences directed against unique sequences in human HLA-A0201 (HLA-A2 allele-specific) and against common sequences conserved among Class I loci (HLA-ABC pan-specific) were designed as short hairpin RNA (shRNA) loop structures and cloned into pLentiLox-DsRed (Figure 3). This HIV-derived lentiviral vector contains multiple cloning sites for insertion of shRNA constructs to be driven by an upstream U6 promoter, and a downstream CMV promoter-DsRed fluorescent protein (marker gene) cassette flanked by loxP sites. FACS analysis for cell surface HLA expression after transient transfection of 293T human embryonic kidney cells with plasmid demonstrated highest silencing activity with either an HLA-A,B,C pan-specific shRNA construct targeting the conserved HLA Class I sequence 5'-GCTACTACAACCAGAGCGAG-3', or with an allele-specific shRNA construct targeting the unique HLA-A0201 sequence 5'-GGATTACATCGCCCTGAAAG-3' (Figure 3, indicated by arrows). These vector constructs were selected for virus construction and further testing. Virus production was performed using a standard third-generation lentiviral system (23), consisting of packaging plasmids (pMD.G encoding VSV-G envelope, pMDLg/p encoding HIV gag-pol, and pRSV-REV encoding HIV rev) co-transfected along with each pLentiLox-DsRed vector plasmid into 293T cells by calcium phosphate precipitation. The resulting viruses deliver both the U6 promoter-driven shRNA cassette and a CMV promoter-driven DsRed fluorescent marker gene cassette upon infection of target cells. Lentiviral vectors expressing only DsRed were also prepared in parallel as negative controls. Virus titers, as determined by FACS analysis for expression of the co-expressed DsRed fluorescent marker protein, were 2 to 10×10^8 transducing units (TU) per mL.

Lentiviral vectors for shRNA targeting of pan-specific and allele-specific HLA sequences were used to transduce naïve 293T cells at increasing multiplicities of infection (MOI; i.e., virus-to-cell ratio). The 293T cell line predominantly expresses HLA Class I antigen HLA-A2 and lower levels of HLA-B7 and -Cw7. FACS analysis of transduced cells showed an increase in mean fluorescence level in the red channel due to co-expression of DsRed. A dose-dependent

reduction in HLA levels due to shRNA expression was detected as a reduction in fluorescence in the green channel after staining with HLA-A2-specific or -ABC pan-specific antibodies conjugated with fluorescein isothiocyanate (FITC). Transduction at higher MOI (10-30) with allele-specific and pan-specific shRNA vectors reduced cell surface expression of HLA-A and HLA-ABC up to 50% and over 80%, respectively, compared to HLA expression levels in cells transduced with lentivirus expressing DsRed only (Table 2, (24)).

Resistance to alloreactive CTL-mediated killing without LAK cell-mediated killing

The effect of lentiviral mediated HLA silencing on CTL-mediated killing of allogeneic cells was tested *in vitro*. Peripheral blood mononuclear cell (PBMC)-derived allo-reactive human effector T lymphocytes (allo-CTL), kindly provided by Dr. Carol Kruse at the Sidney Kimmel Cancer Center, were pre-activated against human stimulator cells expressing the Class I antigens HLA-A2, B44, and C5. Target 293T cells transduced with lentiviral vectors encoding either HLA-A2 allele-specific or HLA-ABC pan-specific shRNA, or with pLentiLox-DsRed control vector were analyzed for sensitivity to cytolysis by incubation with HLA-activated allo-CTL at a ratio of 10:1 (effector: target cell ratio). After 48 hr incubation, the level of interferon-gamma (IFN γ) production by allo-CTLs was measured by ELISA, and the viability of adherent target cells remaining after washing was determined by MTS assay and annexin V staining (24). Target cells transduced with HLA-ABC shRNA and HLA-A0201 shRNA vectors induced significantly less IFN γ production from allo-CTLs, and showed enhanced resistance to alloCTL-mediated killing by both annexin V and MTS assays ($p < 0.05$) compared to cells transduced with control vector expressing DsRed only (24).

A potential limitation of the HLA-silencing approach in the transplant setting is that complete loss of HLA expression may have the undesirable effect of increasing sensitivity to lysis by non-HLA-restricted effectors such as NK and LAK cells. However, we did not observe significant differences in the survival of HLA-ABC shRNA or HLA-A0201 shRNA vector-transduced target cells compared to DsRed only-transduced control cells after incubation with LAK cells derived from the same donor PBMC (24).

Reducing histocompatibility barriers by conditioning the graft, not the host

Our studies demonstrate that lentiviral vectors expressing pan-Class I specific shRNA constructs directed against conserved sequences in HLA-A,B,C, and HLA-A2-specific shRNA constructs can achieve dose-dependent knockdown of HLA levels in human cells, associated with induction of resistance to killing by allo-reactive T effector cells, without incurring significant sensitivity to non-HLA-restricted killer cells.

In concurrent and more recent studies, Blasczyk, Horn, and colleagues have also reported that lentiviral vectors expressing shRNA cassettes targeted against HLA-A and beta2-microglobulin (β_2M) could achieve similar allo-protection in HeLa and immortalized primary B cell lines (25,26). Surrogate assays showed prevention of HLA-A-specific antibody-mediated, complement-dependent cytotoxicity and reduced CD8+ T cell proliferation and IFN γ secretion (25,26). While complete elimination of HLA expression was not achieved, transduced cells showed protection from T cell allo-reactivity by siRNA transduction, and only elicited moderate NK cell reactivity with conditional expression of a lentiviral shRNA vector (26). Our results with lentiviral-mediated delivery of different HLA-A0201-specific and HLA-ABC pan-Class I specific shRNA constructs showed similar results including effectiveness in conferring resistance to allo-reactive CTL by direct measurement of target cell survival (24).

Combining siRNA technology with lentivirus vector-mediated genetic engineering of donor cells or tissues, offers the potential to achieve immunological evasion with less toxicity than currently used non-specific immunosuppressive agents. In this context, it may be advantageous

that HLA knockdown with these particular pan-Class I shRNA constructs did not result in complete loss of expression even at high MOI, as this may avoid sensitivity to non-HLA-restricted killer cell activity. Down-regulation of specific HLA alleles by precise siRNA targeting may expand the histocompatibility and utility of existing donor cells and tissues by nullifying certain classes of HLA sequences and thus making it easier to find matches with the remaining HLA sequences. In the future, issues regarding potential NK /LAK mediated lysis of donor cells with knock-down HLA expression will have to be explored in *in vivo* models of transplantation where the responses of host and donor immune effector cells may be tested.

Global or allele-specific knockdown of HLA in order to effect long-term modulation of the immunogenicity of transplanted cells and tissues, would represent a fundamental shift in the approach to achieving histocompatibility, by engineering the graft rather than immunosuppressing the recipient. While efficient transduction of entire solid organs remains a significant technical hurdle, application of this strategy can be readily envisioned for *ex vivo* transduction of cellular transplants in which HLA matching is a rate-limiting factor, such as bone marrow transplants, skin grafts and pancreatic islet cells. With regard to HSC transplantation in particular, the primary technical goals now are to improve the efficiency of gene transfer and the engraftment of genetically modified hematopoietic progenitor cells to clinically relevant levels. As noted above, lentiviral vectors are a more efficient system for gene transfer to quiescent HSC than previously tested vectors because they encode proteins that permit active import of the viral genome into the nucleus in the G0/G1 phase of the cell cycle (Table 1, Figure 2). However, remaining hurdles to the application of gene therapy for hereditary and other disorders include variable transduction of HSC and gene expression, and immune responses to vectors, therapeutic genes, and allo-antigens in the case of allogeneic transplantation.

Approaches to exploring *in vivo* chemoselection to enhance hematopoietic reconstitution

Clearly, additional complementary strategies for genetic engineering of HSC that would provide a selective advantage for transplanted donor cells and enable successful engraftment with less toxic preparative and immunosuppressive regimens would increase the numbers of individuals to whom this potentially curative therapy could be offered. The rarity and quiescence of HSCs has led to the development of several strategies to amplify these cells either *ex vivo* or *in vivo*. The ability to increase the numbers of HSCs *ex vivo* could increase the efficiency with which stem cells could be modified by expression of shRNA or by corrective therapeutic genes, and would also increase the numbers of cells available for transplantation. Many earlier studies focused on the use of combinations of recombinant cytokines to enhance proliferation of HSCs *ex vivo*. However, concerns were raised that extended culture decreased the re-populating and self-renewal activity of cultured cells (27-29).

As an alternative approach, HSC transduction with vectors carrying drug resistance genes has been tested as a method for achieving positive selection of the transduced cells *in vivo*. The human Multi-Drug Resistance gene (MDR1) or the dihydrofolate reductase (DHFR) gene were introduced into BM cells by oncoretroviral-mediated transduction after expansion in culture with IL-3, SCF, IL-6 for 12 days (30). Transduced cells carrying MDR1 were selected in the presence of Taxol, while DHFR-containing cells were selected with trimetrexate. There was a 100-fold *in vitro* expansion of cells with either of the selective agents; however, engraftment of transplanted DHFR transduced cells was only transient with *in vivo* selection (30). In contrast, BM populations transduced with MDR1 and selected *in vivo* with taxol showed long-term engraftment, but all mice transplanted with these cells developed a myeloproliferative syndrome (30). Another approach for HSC expansion has been the introduction of HoxB4 (31). However, expression of HoxB4 has been associated with the emergence of leukemic

clones in a large animal model (32). Thus, these approaches have been associated with considerable liabilities.

Lentiviral-mediated expression of P140K-MGMT and *in vivo* chemoselection of HSC

A recent advance in positive selection strategies has been to use lentiviral transduction and *in vivo* selection of HSC expressing a modified DNA alkyltransferase, the P140K variant of the MGMT gene (P140K-MGMT) (33). *In vivo* expansion of HSC populations is achieved by administration of benzylguanine (BG) to inhibit endogenous MGMT activity and the nitrosourea BCNU, or other alkylating agents such as temozolomide, to which the P140K-MGMT shows 1000-fold resistance compared with the wild type enzyme (34-37). Transplantation of P140K-MGMT transduced congenic HSC in adult animals followed by successive cycles of BG/BCNU *in vivo* provides selection at the stem cell level and high levels of donor chimerism, without major toxicity (37). Chemo-selection after P140K-MGMT-lentiviral transduction and amplification has also been successfully demonstrated with human NOD/SCID repopulating cells (36,38) and with allogeneic canine CD34+ cells in a large animal model, with sustained multilineage enrichment of transduced repopulating cells (39,40). Similarly, in an adult murine syngeneic transplant model, peripheral blood showed high levels of GFP+ cells in multiple lineages after two cycles of *in vivo* selection (34).

Neonatal models for testing genetic engineering approaches to modify donor stem cells

We are combining the MGMT-based positive selection strategy for *in vivo* amplification of drug-resistant donor cells with transplantation during the neonatal period, when tolerance may be more readily achieved. Our rationale is that the neonatal period is a unique window during immune ontogeny for transplantation of genetically modified allogeneic HSC to achieve long-term engraftment. Neonatal transplantation provides a model system for exploring mechanisms of tolerance induction to neo-antigens, and allogeneic stem cell engraftment. The MGMT-based *in vivo* chemoselection strategy with successive cycles of BG/BCNU will both expand donor stem cells and potentially deplete allo-reactive cells of donor and host origin, reducing the need for toxic ablative or immunosuppressive treatment.

In collaborative studies with Dr Stanton Gerson's laboratory, Case Western Reserve University, we have tested the efficacy of a bifunctional P140K-MGMT-GFP (MAG) lentiviral vector in providing a selective advantage to transduced adult BM HSC after neonatal transplantation and *in vivo* selection with BCNU and BG (Figure 4). The functionality of the vector was first tested in *in vitro* studies of lentivirally transduced 293T cells. Transduced cells and untransduced were cultured with BG/BCNU for 6 days (Figure 5A). While MAG-transduced 293T cells were resistant to BG/BCNU, control untransduced cells showed greater than 90% cell death. Studies in the Gerson laboratory have demonstrated efficient transduction of human CD34+ at low MOI and *in vivo* selection with BCNU (41). Significantly, myeloablation was not required for efficient *in vivo* selection of syngeneic transduced, transplanted cells. The P140K-MGMT mutant is 20-fold more BG-resistant than other variants such as G156A-MGMT (37).

The feasibility of this *in vivo* HSC selection strategy was then assessed in our neonatal transplantation model. BALB/c whole BM was transduced with MAG lentivirus and transplanted into day 2 BALB/c neonates after non-ablative conditioning (Figure 5B). Mice were treated with BG/BCNU at 5 and 11 weeks after birth and flow analysis of GFP expression performed before, and 1 month after, each drug cycle. After two cycles, 39.5% of mononuclear cells in peripheral blood were GFP positive showing successful engraftment and *in vivo* chemoselection of syngeneic GFP+ donor cells without significant toxicity in recipients.

We have also demonstrated the feasibility and efficacy of MAG-lentiviral mediated transduction and *in vivo* chemoselection of transduced allogeneic BM in the neonatal transplant model. BALB/c whole BM was transduced and transplanted into day 2 C57Bl/6XBALB/c F1 neonates after treatment with busulfan and low dose radiation at birth. Two cycles of BG/BCNU were administered 5 and 10 weeks after transplant. Flow analysis of peripheral blood prior to chemoselection demonstrated 1-3% GFP+ cells. However, after two cycles of BG/BCNU up to 37% GFP+ donor cells were seen in peripheral blood (Figure 5C). Levels of donor chimerism were stable thereafter, and no signs of GVHD or immune responses to the GFP neo-antigen were detected in animals observed longitudinally.

This neonatal transplantation model will be a valuable tool for evaluating different and complementary strategies for genetic engineering of HSC. The model will not only be useful for addressing technical hurdles to successful gene therapy and improved safety and applicability of allogeneic transplantation, but will also provide insights into mechanisms of immune tolerance induction during immune ontogeny. We have shown that transplantation in the neonatal period permits stable high-level engraftment of syngeneic or allogeneic HSC with minimal preparative regimens using *in vivo* chemoselection. Further, enrichment of p140-MGMT-GFP (MAG) transduced HSC after either syngeneic or semi-allogeneic neonatal transplant and BG/BCNU selection was achieved without evidence of immune responses to either neo-antigens (GFP) or allo-antigens. In the future, combining approaches including RNAi mediated knockdown of HLA, modulation of immune responses, and strategies to provide selective advantages for transplanted genetically modified HSC or other donor cells hold great promise for the field of tissue transplantation.

Acknowledgments

We would like to acknowledge the researchers in the Kasahara and Gaensler laboratories who have contributed to this work: In the Kasahara laboratory, Dr. Kazunori Haga and Nathan Lemp contributed equally as the primary researchers who performed the studies described in this review; Dr. Christopher R. Logg designed the original shRNA constructs, and supervised their cloning and testing in lentiviral vectors by Jill Nagashima; Dr. Emmanuelle Faure-Kumar performed large-scale vector production; Dr. Renata Stripecke provided assistance in data analysis and helpful discussion, and the UCLA Vector Shared Resource / Core facility provided technical assistance. We would also like to thank our collaborators, Dr. James Cicciarelli and Dr. Robert Mendez at the National Institute of Transplantation (NIT) at St. Vincent's Medical Center in Los Angeles, Dr. German G. Gomez and Dr. Carol Kruse at the Sidney Kimmel Cancer Center in San Diego, and Dr. William McBride and Dr. Richard Gatti at the UCLA Center for Biological Radioprotectors. Work in the Kasahara laboratory was supported in part by the NIAID-funded UCLA Center for Biological Radioprotectors (U19 AI 067769), the NIT Foundation, and the California Institute for Regenerative Medicine (RS1-00402-01).

Researchers in the Gaensler laboratory who have contributed to this work include Dr. Linda Flebbe-Rehwaltd who developed the initial neonatal transplantation model including the non-ablative preparative regimen and *in vivo* chemoselection protocols; Dr. Rustom Falahati performed the *in vitro* chemoselection assay, optimized conditions for lentiviral transduction and transplantation of transduced HSC, and performed FACS and immunological analyses. We would like to thank our collaborators Dr. Stanton Gerson at the Department of Medicine, Division of Hematology-Oncology, Case Western Reserve University, Cleveland, Ohio, who provided the MAG vector, and Dr. William Murphy at the Department of Microbiology and Immunology, University of Nevada, Reno, Nevada for valuable advice. The work in the Gaensler laboratory was supported by NIH 5R21 DK069430-02, NIH 1R01HL082665-01A2, and a UCSF Bridge Funding award.

REFERENCES

1. Sands MS, Barker JE, Vogler C, Levy B, Gwynn B, Galvin N, Sly WS, Birkenmeier E. Treatment of murine mucopolysaccharidosis type VII by syngeneic bone marrow transplantation in neonates. *Lab Invest* 1993;68:676-686. [PubMed: 8515654]
2. Petersdorf EW, Malkki M. Human leukocyte antigen matching in unrelated donor hematopoietic cell transplantation. *Semin Hematol* 2005;42:76-84. [PubMed: 15846573]

3. Petersdorf EW, Hansen JA, Martin PJ, Woolfrey A, Malkki M, Gooley T, Storer B, Mickelson E, Smith A, Anasetti C. Major-histocompatibility-complex class I alleles and antigens in hematopoietic-cell transplantation. *N Engl J Med* 2001;345:1794–1800. [PubMed: 11752355]
4. Tiercy JM, Villard J, Roosnek E. Selection of unrelated bone marrow donors by serology, molecular typing and cellular assays. *Transpl Immunol* 2002;10:215–221. [PubMed: 12216952]
5. Fleischhauer K, Kernan NA, O'Reilly RJ, Dupont B, Yang SY. Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. *N Engl J Med* 1990;323:1818–1822. [PubMed: 2247120]
6. Petersdorf EW, Anasetti C, Martin PJ, Gooley T, Radich J, Malkki M, Woolfrey A, Smith A, Mickelson E, Hansen JA. Limits of HLA mismatching in unrelated hematopoietic cell transplantation. *Blood* 2004;104:2976–2980. [PubMed: 15251989]
7. Sasazuki T, Juji T, Morishima Y, Kinukawa N, Kashiwabara H, Inoko H, Yoshida T, Kimura A, Akaza T, Kamikawaji N, Koda Y, Takaku F. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. Japan Marrow Donor Program. *N Engl J Med* 1998;339:1177–1185. [PubMed: 9780337]
8. Rubinstein P. HLA matching for bone marrow transplantation--how much is enough? *N Engl J Med* 2001;345:1842–1844. [PubMed: 11752363]
9. Vargas-Diez E, Fernandez-Herrera J, Marin A, Camara R, Garcia-Diez A. Analysis of risk factors for acute cutaneous graft-versus-host disease after allogeneic stem cell transplantation. *Br J Dermatol* 2003;148:1129–1134. [PubMed: 12828739]
10. McDonald RA, Smith JM, Ho M, Lindblad R, Ikle D, Grimm P, Wyatt R, Arar M, Liereman D, Bridges N, Harmon W. Incidence of PTLT in pediatric renal transplant recipients receiving basiliximab, calcineurin inhibitor, sirolimus and steroids. *Am J Transplant* 2008;8:984–989. [PubMed: 18416737]
11. Abed N, Casper JT, Camitta BM, Margolis D, Trost B, Orentas R, Chang CC. Evaluation of histogenesis of B-lymphocytes in pediatric EBV-related post-transplant lymphoproliferative disorders. *Bone Marrow Transplant* 2004;33:321–327. [PubMed: 14688820]
12. Freland S, Chambers BJ, Andersson M, Van Kaer L, Ljunggren HG. Rejection of allogeneic and syngeneic but not MHC class I-deficient tumor grafts by MHC class I-deficient mice. *J Immunol* 1998;160:572–579. [PubMed: 9551890]
13. Pollak R, Blanchard JM. Organ donor or graft pretreatment to prolong allograft survival: lessons learned in the murine model. *Transplantation* 2000;69:2432–2439. [PubMed: 10868654]
14. Mhashilkar AM, Doebeis C, Seifert M, Busch A, Zani C, Soo Hoo J, Nagy M, Ritter T, Volk HD, Marasco WA. Intrabody-mediated phenotypic knockout of major histocompatibility complex class I expression in human and monkey cell lines and in primary human keratinocytes. *Gene Ther* 2002;9:307–319. [PubMed: 11938450]
15. Beyer F, Doebeis C, Busch A, Ritter T, Mhashilkar A, Marasco WM, Laube H, Volk HD, Seifert M. Decline of surface MHC I by adenoviral gene transfer of anti-MHC I intrabodies in human endothelial cells-new perspectives for the generation of universal donor cells for tissue transplantation. *J Gene Med* 2004;6:616–623. [PubMed: 15170732]
16. Drukker M, Benvenisty N. The immunogenicity of human embryonic stem-derived cells. *Trends Biotechnol* 2004;22:136–141. [PubMed: 15036864]
17. Bradley JA, Bolton EM, Pedersen RA. Stem cell medicine encounters the immune system. *Nat Rev Immunol* 2002;2:859–871. [PubMed: 12415309]
18. Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet* 2001;2:110–119. [PubMed: 11253050]
19. McManus MT, Petersen CP, Haines BB, Chen J, Sharp PA. Gene silencing using micro-RNA designed hairpins. *Rna* 2002;8:842–850. [PubMed: 12088155]
20. Gonzalez S, Castanotto D, Li H, Olivares S, Jensen MC, Forman SJ, Rossi JJ, Cooper LJ. Amplification of RNAi--targeting HLA mRNAs. *Mol Ther* 2005;11:811–818. [PubMed: 15851019]
21. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JL, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C,

- Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 2003;302:415–419. [PubMed: 14564000]
22. Fischer A, Abina SH, Thrasher A, von Kalle C, Cavazzana-Calvo M. LMO2 and gene therapy for severe combined immunodeficiency. *N Engl J Med* 2004;350:2526–2527. author reply 2526–2527. [PubMed: 15190153]
 23. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998;72:8463–8471. [PubMed: 9765382]
 24. Haga K, Lemp N, Logg CR, Nagashima J, Faure-Kumar E, Gomez GG, Kruse CA, Mendez R, Stripecke R, Kasahara N, Cicciarelli J. Permanent, lowered HLA Class I expression using lentivirus vectors with shRNA constructs: Averting cytotoxicity by alloreactive T lymphocytes. *Transpl Proc* 2006;38:3184–3188.
 25. Figueiredo C, Seltsam A, Blasczyk R. Class-, gene-, and group-specific HLA silencing by lentiviral shRNA delivery. *J Mol Med* 2006;84:425–437. [PubMed: 16520945]
 26. Figueiredo C, Horn PA, Blasczyk R, Seltsam A. Regulating MHC expression for cellular therapeutics. *Transfusion* 2007;47:18–27. [PubMed: 17207226]
 27. Kittler EL, Peters SO, Crittenden RB, Debatis ME, Ramshaw HS, Stewart FM, Quesenberry PJ. Cytokine-facilitated transduction leads to low-level engraftment in nonablated hosts. *Blood* 1997;90:865–872. [PubMed: 9226188]
 28. Traycoff CM, Cornetta K, Yoder MC, Davidson A, Srouf EF. Ex vivo expansion of murine hematopoietic progenitor cells generates classes of expanded cells possessing different levels of bone marrow repopulating potential. *Exp Hematol* 1996;24:299–306. [PubMed: 8641356]
 29. Peters SO, Kittler EL, Ramshaw HS, Quesenberry PJ. Murine marrow cells expanded in culture with IL-3, IL-6, IL-11, and SCF acquire an engraftment defect in normal hosts. *Exp Hematol* 1995;23:461–469. [PubMed: 7536685]
 30. Bunting KD, Galipeau J, Topham D, Benaim E, Sorrentino BP. Transduction of murine bone marrow cells with an MDR1 vector enables ex vivo stem cell expansion, but these expanded grafts cause a myeloproliferative syndrome in transplanted mice. *Blood* 1998;92:2269–2279. [PubMed: 9746764]
 31. Cellot S, Krosi J, Chagraoui J, Meloche S, Humphries RK, Sauvageau G. Sustained in vitro trigger of self-renewal divisions in Hoxb4hiPbx1(10) hematopoietic stem cells. *Exp Hematol* 2007;35:802–816. [PubMed: 17577929]
 32. Zhang XB, Beard BC, Trobridge GD, Wood BL, Sale GE, Sud R, Humphries RK, Kiem HP. High incidence of leukemia in large animals after stem cell gene therapy with a HOXB4-expressing retroviral vector. *J Clin Invest* 2008;118:1502–1510. [PubMed: 18357342]
 33. Davis BM, Roth JC, Liu L, Xu-Welliver M, Pegg AE, Gerson SL. Characterization of the P140K, PVP(138-140)MLK, and G156A O6-methylguanine-DNA methyltransferase mutants: implications for drug resistance gene therapy. *Hum Gene Ther* 1999;10:2769–2778. [PubMed: 10584923]
 34. Sawai N, Zhou S, Vanin EF, Houghton P, Brent TP, Sorrentino BP. Protection and in vivo selection of hematopoietic stem cells using temozolomide, O6-benzylguanine, and an alkyltransferase-expressing retroviral vector. *Mol Ther* 2001;3:78–87. [PubMed: 11162314]
 35. Persons DA, Allay ER, Sawai N, Hargrove PW, Brent TP, Hanawa H, Nienhuis AW, Sorrentino BP. Successful treatment of murine beta-thalassemia using in vivo selection of genetically modified, drug-resistant hematopoietic stem cells. *Blood* 2003;102:506–513. [PubMed: 12663444]
 36. Zielske SP, Reese JS, Lingas KT, Donze JR, Gerson SL. In vivo selection of MGMT(P140K) lentivirus-transduced human NOD/SCID repopulating cells without pretransplant irradiation conditioning. *J Clin Invest* 2003;112:1561–1570. [PubMed: 14617757]
 37. Bowman JE, Reese JS, Lingas KT, Gerson SL. Myeloablation is not required to select and maintain expression of the drug-resistance gene, mutant MGMT, in primary and secondary recipients. *Mol Ther* 2003;8:42–50. [PubMed: 12842427]
 38. Pollok KE, Hartwell JR, Braber A, Cooper RJ, Jansen M, Ragg S, Bailey BJ, Erickson LC, Kreklau EL, Williams DA. In vivo selection of human hematopoietic cells in a xenograft model using combined pharmacologic and genetic manipulations. *Hum Gene Ther* 2003;14:1703–1714. [PubMed: 14670122]
 39. Neff T, Horn PA, Peterson LJ, Thomasson BM, Thompson J, Williams DA, Schmidt M, Georges GE, von Kalle C, Kiem HP. Methylguanine methyltransferase-mediated in vivo selection and

- chemoprotection of allogeneic stem cells in a large-animal model. *J Clin Invest* 2003;112:1581–1588. [PubMed: 14617759]
40. Neff T, Beard BC, Peterson LJ, Anandakumar P, Thompson J, Kiem HP. Polyclonal chemoprotection against temozolomide in a large-animal model of drug resistance gene therapy. *Blood* 2005;105:997–1002. [PubMed: 15494421]
41. Zielske SP, Gerson SL. Lentiviral transduction of P140K MGMT into human CD34(+) hematopoietic progenitors at low multiplicity of infection confers significant resistance to BG/BCNU and allows selection in vitro. *Mol Ther* 2002;5:381–387. [PubMed: 11945064]

Figure 1. Targeting HLA Class I to reduce graft immunogenicity

Three potential mechanisms for allograft rejection of graft cell A, resulting in its elimination are depicted: 1) direct antigen recognition, in which T cell receptors (TCR) on host T cells recognize intact donor HLA molecules on the graft cells as non-self, presumably because their three-dimensional structure resembles a self MHC bound to a foreign peptide ("molecular mimicry"), 2) peptides derived from donor HLA molecules are presented by host antigen-presenting cells (APC) as foreign antigens, 3) host antibodies against donor HLA bind and initiate graft damage through antibody-dependent cellular toxicity and complement activation. Graft cell B depicts the proposed scenario if HLA expression is silenced: although co-stimulatory molecules such as CD80 (2nd signal) might be displayed, none of the above mechanisms would be activated in the absence of HLA on the donor-derived graft cells. This could lead to prolonged survival by making the graft cells invisible to allo-reactive immune responses, but could also incur MHC-non-restricted killing by host NK or LAK cells.

Figure 2. Molecular events associated with retrovirus or lentivirus vector -mediated transduction (asterisks denote aspects specific to lentiviral vectors). 1: Virion adsorption via interaction between viral envelope protein and cell surface receptor (* lentiviral vectors, and in some cases retroviral vectors, can be pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) envelope, allowing broad tropism by binding directly to phospholipids). 2: Virus-cell lipid membrane fusion, allowing entry of viral nucleocapsid complex into cytoplasm. 3: Reverse transcription of viral genomic RNA (single line) to double-stranded DNA (double lines), U3 and U5 sequences duplicated at 5' and 3' ends, respectively, to convert R-U5 and U3-R into matching long terminal repeat (LTR) sequences flanking viral genome. 4: Entry into cell nucleus, either by passive diffusion upon nuclear membrane breakdown during mitosis (oncoretrovirus) or *active uptake by recognition of nuclear localization signal (NLS; lentivirus). 5: Permanent integration of proviral DNA into host cell chromosome, resulting in stable long-term transduction.

Sequences specific to A*020101 allele of human MHC I:

A2-1: GGATTACATCGCCCTGAAAG ←
 A2-2: GCAGGAGGGTCCGGAGTATT
 A2-3: GGACGGGGAGACACGGAAAG
 A2-4: GAAAGTGAAGGCCCACTCA

Sequences common to the most frequent alleles of human MHC I - A, B and C loci :

ABC-1: GATACCTGGAGAACGGGAAG
 ABC-2: GCTGTGGTGGTGCCTTCTGG ←
 ABC-3: GCTACTACAACCAGAGCGAG
 ABC-4: GTGGCTCCGCAGATACCTG

Figure 3. Lentiviral vector constructs for short hairpin RNAs directed against HLA

The basic lentiviral vector pLentiLox-DsRed construct contains a U6 promoter that can drive expression of a short hairpin siRNA (shRNA; sense siRNA-loop-antisense siRNA) sequence, and a downstream CMV promoter-driven red fluorescent marker gene (DsRed) cassette. LTR: lentiviral long terminal repeat, Ψ : packaging signal. Shown below are the allele-specific siRNA sequences (designed against HLA- A2.1 (A*020101 allele)) and pan-specific siRNA sequences (against conserved regions in HLA-A, B, C) that were designed and tested in the pLentiLox vector. Arrows indicate the sequences showing the best knockdown activity that were selected for further testing.

Figure 4. *In vitro* gene delivery of MGMT into HSC followed by transplantation and *in vivo* chemoselection

Donor HSC are isolated (Step 1) and transduced *in vitro* with lentivirus containing the MGMT^{P140K}-GFP transgene (MAG vector) resulting in integration into chromosomal DNA (Step 2). Subsequently, neonatal mice are preconditioned with a non-ablative regimen (Step 3) and MAG transduced HSC are delivered via intravenous injection (Step 4). Following engraftment, *in vivo* chemoselection is performed by delivery of BG followed by BCNU (Step 5). Initially the graft consists of a small numbers of MAG-transduced HSC. Sequential chemoselection (Step 6) results in apoptosis of untransduced HSCs and enrichment of MAG-transduced HSC. Enriched MAG- transduced HSC expand and repopulate all hematopoietic lineages.

Figure 5. *In vitro* and *In vivo* chemoselection of syngenic and allogeneic HSC

(A) Lentivirus MAG transduced (> 90% expressing MGMT^{P140K}) or untransduced 293 T cells were incubated with 10 uM BG and 50 uM BCNU for 6 days. Viability of cells on Day 6 following treatment was evaluated by using the MTS assay (Promega). Percent survival is shown for each group as compared to untransduced control cells without drug treatment. (B) BALB/c whole BM cells (5×10^5) were transduced overnight after prestimulation and transplanted into BALB/c neonates preconditioned with a non-myleoablative regimen. BG (30mg/kg)/BCNU (7.5mg/kg) was administered 5 and 11 weeks post transplant. Flow analysis of peripheral blood was performed immediately prior to the initiation of chemoselection (light gray), and at 11 (dark gray), and 15 weeks post transplant (Black curve). (C) BALB/c whole BM cells (1.7×10^6) were transduced by spinoculation and transplanted into 2 day-old C57BL/6 X BALB/c F1 neonates preconditioned with a non-myleoablative regimen. BG (30mg/kg)/BCNU (7.5mg/kg) was administered 5 and 10 weeks post transplant. Flow analysis of peripheral blood was performed immediately prior to the initiation of chemoselection (light gray curve), and at 10 weeks (dark gray curve), and 15 weeks (black curve) post transplant.

Table 1

Advantages of using lentivirus-based vectors for efficient and stable gene-silencing of HLA expression in human cells

Advantages of using lentivirus-based vectors for suppression of HLA expression	
<ul style="list-style-type: none">••••	Efficient transduction of growth-arrested cell lines and quiescent cells e.g. hematopoietic stem cells
	Permanent integration into the genomic DNA, thereby enabling long-term modification of HLA phenotype with a single procedure
	No possibility that expression of native viral genes might lead to immunological rejection of cells transduced by replication-defective lentivirus vectors <i>in vivo</i>
	siRNA molecules that target HLA-transcripts do not encode foreign proteins and therefore cannot become a target for immune attack

Table 2

Studies using lentivirus-based vectors for efficient and stable gene-knockdown of HLA expression in human cells

Targeted gene/allele	Vector	Transduced cells	Assay	Result	Ref.
HLA heavy chain β 2-microglobulin (β 2m)	Drug-inducible expression system: pLV-tTR-KRAB CL1.THPC CLIN.THPC	HeLa B-LCL primary monocytes	<ul style="list-style-type: none"> Analysis of HLA expression by FACS Quantification of transcripts by qRT-PCR T-cell proliferation assay Complement-dependent cytotoxicity assay NK cell cytotoxicity assay 	<ul style="list-style-type: none"> Stable and drug-inducible silencing of HLA-A or HLA class I antigens by up to 90% Effective prevention of antibody-mediated cell lysis and CD8⁺T-cell response Effective inhibition of NK-cell-mediated lysis by residual HLA expression 	[26]
HLA-A-A0201 (HLA-A2 allele-specific) HLA-ABC pan-specific	pLentiLox-DsRed	HEK293T	<ul style="list-style-type: none"> Analysis of HLA expression by FACS AlloCTL/LAK cytotoxicity assay 	<ul style="list-style-type: none"> Efficient and dose-dependent reduction in HLA surface expression Resistance to killing by alloCTL No significant sensitivity to non-HLA-restricted killer cells 	[24]
HLA heavy chain β 2-microglobulin (β 2m)	BLOCK-iT Lentiviral RNAi Expression System: pENTR/U6 entry vector pLenti6/Block-iT-DEST	HeLa B-LCL K562	<ul style="list-style-type: none"> Analysis of HLA expression by FACS Quantification of transcripts by qRT-PCR Interferon-γ stimulation assay T cell assay Complement-dependent cytotoxicity assay NK cell cytotoxicity assay 	<ul style="list-style-type: none"> HLA class I suppression in a class- and gene-specific way and on the HLA allele level. HLA class I suppression under inflammatory conditions Effective prevention of antibody-mediated cell lysis and CD8⁺T cell response Effective inhibition of NK-cell-mediated lysis by residual HLA expression 	[25]