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Suppression of HLA expression by lentivirus-mediated gene transfer of siRNA cassettes and in vivo chemoselection to enhance hematopoietic stem cell transplantation

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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

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killing. 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-O^6-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 Human leukocyte antigen (HLA) · Major histocompability complex (MHC) · Short hairpin-type RNAi (shRNA) · RNA interference (RNAi) · O^6-methylguanine-DNA-methyltransferase (MGMT) · $MGMT^{P140K}$-2A-GFP lentivirus vector (MAG) · Busulfan (BU) · Benzylguanine (BG) · BCNU-1 · 3-Bis(2-chloroethyl1-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 versus 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 mis-matched 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 (SCID) 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

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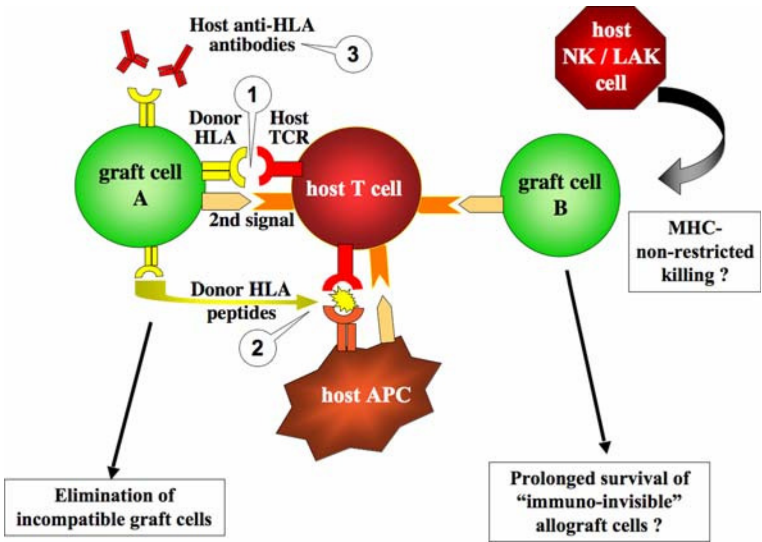

Fig. 1 Targeting HLA Class I to reduce graft immunogenicity. Three potential mechanisms for allograft rejection of graft cell A, resulting in its elimination are depicted: (1) direct antigen recognition, in which T cell receptors (TCR) on host T cells recognize intact donor HLA molecules on the graft 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 (second 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

71 host by suppression/ablation of the host immune system using irradiation or potent
72 chemotherapy.

73 Limitations for application of allogeneic transplantation: HLA matching

74 The most important loci for graft survival are the HLA Class I antigens A and B, and the
75 Class II antigen DR (Fig. 1). However, HLA are highly polymorphic with more than 220,
76 460, 110, and 360 molecularly defined epitopes for HLA-A, B, C, and DR, respectively.
77 Mismatching of the serological antigens increases the probability of graft failure [2–4].
78 Even with serological cross-matching, small molecular genetic differences may result in
79 graft rejection [5–7]. Further, GVHD, associated with HLA antigen mismatching
80 between donor and recipient, results in considerable morbidity and mortality [8, 9].
81 Despite the growing size of national and international registries, there continues to be a
82 lack of suitable donors for both stem cell and organ donation. Procuring suitable HLA-
83 matched donors represents the major limitation to the field of organ and stem cell trans-
84 plantation. Finally, even with well-matched HLA-A, B, and DR antigens, potent immu-
85 nosuppressive regimens are required. Thus, for patients, there is a fine balance between
86 "too little" immunosuppression and graft rejection, or "too much" immunosuppression
87 resulting in infection and toxicity to the recipient, and in some cases, leading to post-
88 transplant malignancies [10, 11].

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

90 We have been pursuing a novel strategy for genetically modifying graft cells to achieve
91 long-term suppression of cellular proteins that elicit or potentiate host immune responses
92 against mismatched donor antigens. In principle, modifications to reduce or eliminate
93 expression of specific HLA expression on donor cells would reduce alloreactive immune
94 responses, the need for full HLA matching, and the need to suppress the host immune sys-
95 tem. The goal of this approach is to achieve down-regulation of HLA expression sufficient
96 to alleviate graft rejection without deleteriously impacting immune responses against for-
97 eign pathogens. Support for the concept that long-term suppression of HLA may enable
98 donor cells to evade host immune responses comes from studies showing that grafts from
99 MHC Class I and Class II "knockout" mice survive longer than controls [12, 13]. However,
100 antibody-based strategies such as graft pre-treatment with haplotype-specific anti-Ia mAb,
101 haplotype-specific immunoconjugates, or mAb directed against graft antigen presenting
102 cells (APC), adhesion or costimulation molecules, were found to have little efficacy in pro-
103 moting acceptance of allografts in immunocompetent hosts [13].
104 Virus-based gene transfer vectors, such as lentivirus vectors, adeno-associated virus
105 (AAV) vectors, and helper-dependent adenovirus vectors may be used to transduce graft
106 cells with sequences encoding small interfering RNAs (siRNAs), anti-sense mRNA, and
107 ribozymes directed against critical immune recognition molecules or immunostimulatory
108 factors. Engineering the graft using these approaches could decrease donor cell immunoge-
109 nicity and increase the pool of unrelated BM donors, while reducing the need for toxic
110 myeloablative pre-conditioning or immunosuppressive regimens.
111 Recent studies have demonstrated that adenoviral-mediated expression of an anti-human
112 MHC I single-chain intrabody achieved 'phenotypic knockout' of Class I HLA in human
113 primary keratinocytes and in endothelial (HUVEC) cells [14, 15]. Intrabody-transduced
114 cells were protected from lysis by sensitized allogeneic cytotoxic T lymphocytes (CTLs),
115 while control cells from the same donor were not [15], establishing proof-of-concept for
116 targeted inhibition of MHC expression in reducing immunogenicity of adult tissue allo-
117 grafts. However, in terms of practical implementation, adenoviral-mediated approaches are
118 unlikely to have long term benefits for graft survival as adenoviruses are nonintegrating
119 vectors that direct short-lived expression and are highly immunogenic due to the leaky
120 expression of viral proteins. Further, the synthetic anti-MHC intrabody protein itself also
121 represents a neo-antigen that may be immunogenic.

122 Application of siRNA-based methods for knockdown of HLA expression

123 In contrast to adenoviral vectors, the use of oncoretrovirus- or lentivirus-based gene trans-
124 fer vectors enables long-term transduction of donor HSC and expression of sequences
125 encoding siRNAs, anti-sense mRNA, or ribozymes directed against immune recognition
126 molecules such as HLA, or co-stimulatory molecules such as CD80. These vectors could
127 also be used for gene delivery of specific ubiquinating enzymes, dominant-negative inhibi-
128 tory proteins, and transmembrane or secreted immunosuppressive factors. The effect of
129 such manipulations will be to decrease immunogenicity of donor cells and associated host-
130 and graft-mediated immune responses. A potential limitation of this approach is that com-
131 plete silencing of HLA may render donor cells susceptible to recognition and attack by
132 non-HLA-restricted effector cells such as natural killer (NK) and lymphokine-activated
133 killer (LAK) cells. It may be necessary to modulate siRNA-mediated silencing and HLA
134 expression to a level that does not allow recognition by alloreactive T cells and yet will not

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135 elicit NK-mediated lysis. Alternatively, precise knockdown of specific HLA alleles may be
136 used to nullify potential mis-matches. For example, lentiviral-mediated class- and gene-
137 specific silencing of HLA would broaden the application for transplantation of existing
138 human embryonic stem cell (hESC) pools in future regenerative medicine approaches by
139 reducing the problem of HLA mismatches in recipients of hESC-derived grafts [16, 17].
140 RNA interference (RNAi) is a potent genetic tool for silencing gene expression that trig-
141 gers post-transcriptional degradation of homologous transcripts through a multi-step mech-
142 anism involving double-stranded siRNA [18, 19]. Recently, Cooper, Rossi and colleagues
143 demonstrated that T cells stably transfected with plasmids containing siRNA expression
144 cassettes showed HLA down-regulation that provided protection from cytolysis even when
145 the transfected cells were loaded with a target peptide and challenged with peptide-specific
146 CTLs [20]. However, these stable transformants expressing high copy numbers of siRNA
147 could only be isolated by co-transfection and selection with antibiotic resistance genes in
148 an immortalized Jurkat T cell line. This methodology is not feasible for hematopoietic pro-
149 genitors and particularly quiescent stem cells. Thus, our focus has been to use lentivirus-
150 based vectors, capable of highly efficient transduction of quiescent cells, including HSC.
151 Permanent integration into the host cell genome, provides the potential for long-term sup-
152 pression of HLA in adult cell and organ transplantation.

153 Advantages of Lentiviral-based approaches for HLA-silencing

154 In contrast to adenovirus, AAV, and non-viral gene transfer approaches, both oncoretrovi-
155 ral vectors and lentiviral vectors have the capacity to integrate into the host genome and
156 direct stable gene expression. Onco-retroviral vectors remain one of the most frequently
157 utilized gene delivery modalities in clinical trials to date. During the retroviral life cycle,
158 the reverse transcriptase converts the viral RNA genome to double-stranded DNA, which is
159 then permanently integrated into the chromosomes of the host cell, enabling long-term
160 gene expression. Since native coding sequences have been completely replaced by thera-
161 peutic genes of interest there is no possibility that expression of native viral genes might
162 lead to immunological rejection of cells transduced by replication-defective retrovirus vec-
163 tors. Progressive understanding of the characteristic limitations of MLV-based vectors has
164 spurred numerous improvements in vector production and transduction methods, particu-
165 larly for ex vivo applications, and has led to their more judicious application to relevant
166 clinical scenarios. In this regard, arguably one of the leading successes to date has been the
167 amelioration of X-linked SCID; however, enthusiasm regarding this success has been tem-
168 pered by sobering reminders of the potential for risk, as well as benefit, of viral-mediated
169 gene integration into host chromatin [21, 22].
170 Among the most promising basic advances in gene delivery vector technologies in
171 recent years has been the development of lentivirus-based vector systems. Lentiviruses are
172 complex retroviruses that contain additional regulatory and pathogenicity-enhancing
173 "accessory" genes in addition to the *gag*, *pol*, and *env* structural proteins classically
174 expressed by oncoretroviruses (Table 1). Although the overall life cycle of lentiviruses is
175 similar to that of oncoretroviruses, there are several major differences between these vec-
176 tors. Most notably, oncoretroviruses such as MLV can only transduce cells that divide
177 shortly after infection, because the MLV pre-integration complex cannot achieve chromo-
178 somal integration in the absence of nuclear envelope breakdown during mitosis.
179 In contrast, lentiviruses can infect non-proliferating cells, owing to the karyophilic prop-
180 erties of the lentiviral pre-integration complex, which allows recognition by the cell nuclear
181 import machinery (Table 1; Fig. 2). Correspondingly, lentiviral vectors can transduce cell

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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
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 in vivo
siRNA molecules that target HLA-transcripts do not encode foreign proteins and therefore cannot become a target for immune attack

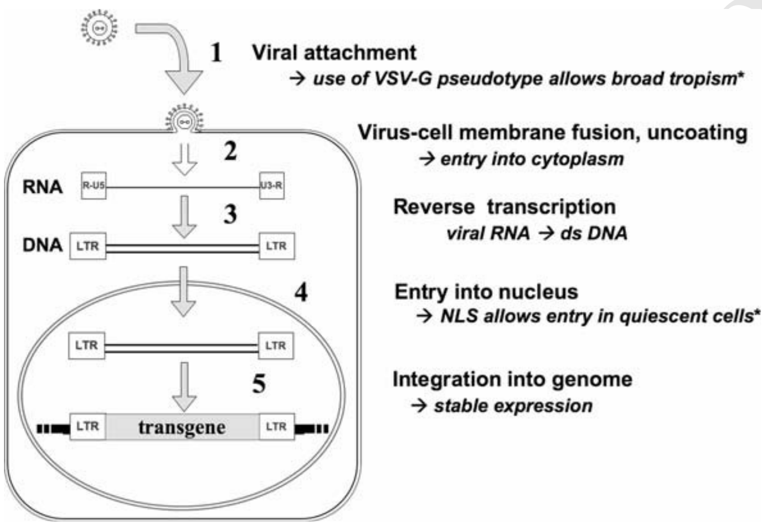

Fig. 2 Molecular events associated with retrovirus or lentivirus vector -mediated transduction (asterisks denote aspects specific to lentiviral vectors). (1) Virion adsorption via interaction between viral envelope protein and cell surface receptor (*lentiviral vectors, and in some cases retroviral vectors, 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

182 lines that are growth-arrested in culture, as well as terminally differentiated primary cells
183 including hematopoietic stem cells, neurons, hepatocytes, cardiomyocytes, endothelium,
184 alveolar pneumocytes, keratinocytes, and dendritic cells [18–26]. Hence, there is increasing
185 interest in the development of vector systems based on a wide variety of lentiviruses,
186 including human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV),
187 feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). We have
188 focused primarily on HIV-based lentiviral vectors for permanent integration of transgenes
189 to achieve long-term modification of cellular phenotype, as this technology has progressed
190 the most rapidly and is already in clinical trials.

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191 Development of lentiviral vectors for silencing of HLA Class I expression

192 To test two different targets for HLA silencing, candidate siRNA sequences directed
193 against unique sequences in human HLA-A0201 (HLA-A2 allele-specific) and against
194 common sequences conserved among Class I loci (HLA-ABC pan-specific) were designed
195 as short hairpin RNA (shRNA) loop structures and cloned into pLentiLox-DsRed (Fig. 3).
196 This HIV-derived lentiviral vector contains multiple cloning sites for insertion of shRNA
197 constructs to be driven by an upstream U6 promoter, and a downstream CMV promoter-
198 DsRed fluorescent protein (marker gene) cassette flanked by loxP sites. FACS analysis for
199 cell surface HLA expression after transient tranfection of 293T human embryonic kidney
200 cells with plasmid demonstrated highest silencing activity with either an HLA-A,B,C pan-
201 specific shRNA construct targeting the conserved HLA Class I sequence 5′-
202 GCTACTACAACCAGAGCGAG-3′, or with an allele-specific shRNA construct targeting
203 the unique HLA-A0201 sequence 5′-GGATTACATCGCCCTGAAAG-3′ (Fig. 3, indi-
204 cated by arrows). These vector constructs were selected for virus construction and further
205 testing. Virus production was performed using a standard third-generation lentiviral system
206 (23), consisting of packaging plasmids (pMD.G encoding VSV-G envelope, pMDLg/p
207 encoding HIV gag–pol, and pRSV-REV encoding HIV rev) co-transfected along with each
208 pLentiLox-DsRed vector plasmid into 293T cells by calcium phosphate precipitation. The
209 resulting viruses deliver both the U6 promoter-driven shRNA cassette and a CMV pro-
210 moter-driven DsRed fluorescent marker gene cassette upon infection of target cells. Lentiv-
211 iral vectors expressing only DsRed were also prepared in parallel as negative controls.
212 Virus titers, as determined by FACS analysis for expression of the co-expressed DsRed
213 fluorescent marker protein, were 2 to 10 × 10 e8 transducing units (TU)/ml.
214 Lentiviral vectors for shRNA targeting of pan-specific and allele-specific HLA
215 sequences were used to transduce naïve 293T cells at increasing multiplicities of infection
216 (MOI; i.e., virus-to-cell ratio). The 293T cell line predominantly expresses HLA Class I
217 antigen HLA-A2 and lower levels of HLA-B7 and -Cw7. FACS analysis of transduced
218 cells showed an increase in mean fluorescence level in the red channel due to co-expression
219 of DsRed. A dose-dependent reduction in HLA levels due to shRNA expression was
220 detected as a reduction in fluorescence in the green channel after staining with HLA-A2-
221 specific or -ABC pan-specific antibodies conjugated with fluorescein isothiocyanate. Trans-
222 duction at higher MOI [10–30] with allele-specific and pan-specific shRNA vectors
223 reduced cell surface expression of HLA-A and HLA-ABC up to 50% and over 80%,
224 respectively, compared to HLA expression levels in cells transduced with lentivirus
225 expressing DsRed only (Table 2, [24]).

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

227 The effect of lentiviral mediated HLA silencing on CTL-mediated killing of allogeneic
228 cells was tested in vitro. Peripheral blood mononuclear cell (PBMC)-derived allo-reactive
229 human effector T lymphocytes (allo-CTL), kindly provided by Dr. Carol Kruse at the Sid-
230 ney Kimmel Cancer Center, were pre-activated against human stimulator cells expressing
231 the Class I antigens HLA-A2, B44, and C5. Target 293T cells transduced with lentiviral
232 vectors encoding either HLA-A2 allele-specific or HLA-ABC pan-specific shRNA, or with
233 pLentiLox-DsRed control vector were analyzed for sensitivity to cytolysis by incubation
234 with HLA-activated allo-CTL at a ratio of 10:1 (effector: target cell ratio). After 48 h incu-
235 bation, the level of interferon-gamma (IFNγ) production by allo-CTLs was measured by

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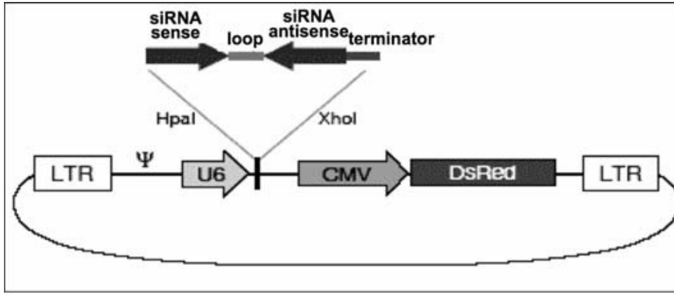

Sequences specific to A*020101 allele of human MHC I:

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

Fig. 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

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 IFNγ 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.

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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 β2-microglobulin (β2m)	Drug-inducible expression system: pLV-tTR-KRAB CL1.THPC CL1N.THPC	HeLa B-LCL primary monocytes	Analysis of HLA expression by FACS Quantification of transcripts by qRT-PCR T-cell proliferation assay Complement-dependent cytotoxicity assay NK cell cytotoxicity assay	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	Analysis of HLA expression by FACS AlloCTL/LAK cytotoxicity assay	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 β2-microglobulin (β2 m)	BLOCK-iT Lentiviral RNAi Expression System: pENTR/U6 entry vector pLenti6/ Block-iT-DEST	HeLa B-LCL K562	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	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]

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254 In concurrent and more recent studies, Blasczyk, Horn, and colleagues have also
255 reported that lentiviral vectors expressing shRNA cassettes targeted against HLA-A and
256 beta2-microglobulin (β_2 M) could achieve similar allo-protection in HeLa and immortal-
257 ized primary B cell lines [25, 26]. Surrogate assays showed prevention of HLA-A-specific
258 antibody-mediated, complement-dependent cytotoxicity, and reduced CD8+ T cell prolifer-
259 ation and IFNγ secretion [25, 26]. While complete elimination of HLA expression was not
260 achieved, transduced cells showed protection from T cell allo-reactivity by siRNA
261 transduction, and only elicited moderate NK cell reactivity with conditional expression of a
262 lentiviral shRNA vector [26]. Our results with lentiviral-mediated delivery of different
263 HLA-A0201-specific and HLA-ABC pan-Class I specific shRNA constructs showed simi-
264 lar results including effectiveness in conferring resistance to allo-reactive CTL by direct
265 measurement of target cell survival [24].
266 Combining siRNA technology with lentivirus vector-mediated genetic engineering of
267 donor cells or tissues, offers the potential to achieve immunological evasion with less toxic-
268 ity than currently used non-specific immunosuppressive agents. In this context, it may be
269 advantageous that HLA knockdown with these particular pan-Class I shRNA constructs did
270 not result in complete loss of expression even at high MOI, as this may avoid sensitivity to
271 non-HLA-restricted killer cell activity. Down-regulation of specific HLA alleles by precise
272 siRNA targeting may expand the histocompatibility and utility of existing donor cells and
273 tissues by nullifying certain classes of HLA sequences and thus making it easier to find
274 matches with the remaining HLA sequences. In the future, issues regarding potential NK/
275 LAK mediated lysis of donor cells with knock-down HLA expression will have to be
276 explored in in vivo models of transplantation where the responses of host and donor
277 immune effector cells may be tested.
278 Global or allele-specific knockdown of HLA in order to effect long-term modulation of
279 the immunogenicity of transplanted cells and tissues would represent a fundamental shift in
280 the approach to achieving histocompatibility, by engineering the graft rather than immuno-
281 suppressing the recipient. While efficient transduction of entire solid organs remains a sig-
282 nificant technical hurdle, application of this strategy can be readily envisioned for ex vivo
283 transduction of cellular transplants in which HLA matching is a rate-limiting factor, such as
284 BM transplants, skin grafts, and pancreatic islet cells. With regard to HSC transplantation
285 in particular, the primary technical goals now are to improve the efficiency of gene transfer
286 and the engraftment of genetically modified hematopoietic progenitor cells to clinically rel-
287 evant levels. As noted above, lentiviral vectors are a more efficient system for gene transfer
288 to quiescent HSC than previously tested vectors because they encode proteins that permit
289 active import of the viral genome into the nucleus in the G0/G1 phase of the cell cycle
290 (Table 1; Fig. 2). However, remaining hurdles to the application of gene therapy for hered-
291 itary and other disorders include variable transduction of HSC and gene expression, and
292 immune responses to vectors, therapeutic genes, and allo-antigens in the case of allogeneic
293 transplantation.

294 Approaches to exploring in vivo chemoselection to enhance hematopoietic reconstitution

295 Clearly, additional complementary strategies for genetic engineering of HSC that would
296 provide a selective advantage for transplanted donor cells and enable successful engraft-
297 ment with less toxic preparative and immunosuppressive regimens would increase the
298 numbers of individuals to whom this potentially curative therapy could be offered. The rar-
299 ity and quiescence of HSCs has led to the development of several strategies to amplify
300 these cells either ex vivo or in vivo. The ability to increase the numbers of HSCs ex vivo

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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 1,000-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

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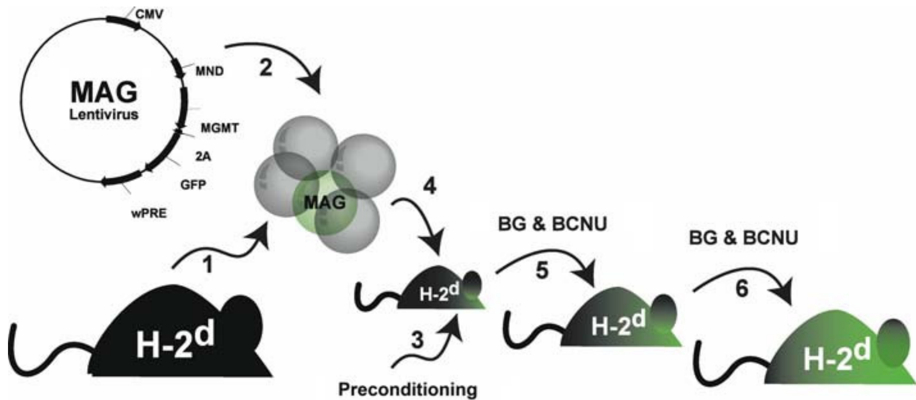

Fig. 4 In vitro gene delivery of MGMT into HSC followed by transplantation and in vivo chemoselection. Donor HSC are isolated (Step 1) and transduced in vitro with lentivirus containing the MGMT^{P140K}–GFP transgene (MAG vector) resulting in integration into chromosomal DNA (Step 2). Subsequently, neonatal mice are pre-conditioned 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

346 donor and host origin, reducing the need for toxic ablative or immunosuppressive treat-
347 ment.
348 In collaborative studies with Dr. Stanton Gerson's Laboratory, Case Western Reserve
349 University, we have tested the efficacy of a bifunctional P140K-MGMT-GFP (MAG) len-
350 tiviral vector in providing a selective advantage to transduced adult BM HSC after neonatal
351 transplantation and in vivo selection with BCNU and BG (Fig. 4). The functionality of the
352 vector was first tested in in vitro studies of lentivirally transduced 293T cells. Transduced
353 cells and untransduced were cultured with BG/BCNU for 6 days (Fig. 5a). While MAG-
354 transduced 293T cells were resistant to BG/BCNU, control untransduced cells showed
355 greater than 90% cell death. Studies in the Gerson laboratory have demonstrated efficient
356 transduction of human CD34+ at low MOI and in vivo selection with BCNU [41]. Signifi-
357 cantly, myeloablation was not required for efficient in vivo selection of syngeneic trans-
358 duced, transplanted cells. The P140K-MGMT mutant is 20-fold more BG-resistant than
359 other variants such as G156A-MGMT [37].
360 The feasibility of this in vivo HSC selection strategy was then assessed in our neonatal
361 transplantation model. BALB/c whole BM was transduced with MAG lentivirus and trans-
362 planted into Day 2 BALB/c neonates after non-ablative conditioning (Fig. 5b). Mice were
363 treated with BG/BCNU at 5 and 11 weeks after birth and flow analysis of GFP expression
364 performed before, and 1 month after, each drug cycle. After two cycles, 39.5% of mononu-
365 clear cells in peripheral blood were GFP positive showing successful engraftment and
366 in vivo chemoselection of syngeneic GFP+ donor cells without significant toxicity in recip-
367 ients.
368 We have also demonstrated the feasibility and efficacy of MAG-lentiviral mediated
369 transduction and in vivo chemoselection of transduced allogeneic BM in the neonatal trans-
370 plant model. BALB/c whole BM was transduced and transplanted into Day 2 C57Bl/
371 6XBALB/c F1 neonates after treatment with busulfan and low dose radiation at birth. Two
372 cycles of BG/BCNU were administered 5 and 10 weeks after transplant. Flow analysis of

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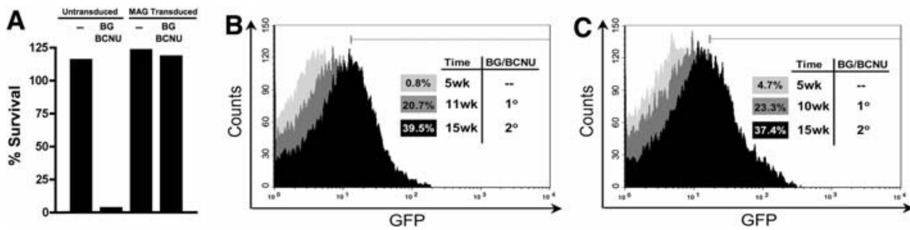

Fig. 5 In vitro and in vivo chemoselection of syngenic and allogeneic HSC. **a** Lentivirus MAG transduced (> 90% expressing MGMT$^{P140\ K}$) or untransduced 293 T cells were incubated with 10 μM BG and 50 μM BCNU for 6 days. Viability of cells on Day 6 following treatment was evaluated by using the 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 pre-stimulation and transplanted into BALB/c neonates pre-conditioned with a non-myleoablative regimen. BG (30 mg/kg)/ BCNU (7.5 mg/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 pre-conditioned with a non-myleoablative regimen. BG (30 mg/kg)/BCNU (7.5 mg/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

373 peripheral blood prior to chemoselection demonstrated 1–3% GFP+ cells. However, after
374 two cycles of BG/BCNU up to 37% GFP+ donor cells were seen in peripheral blood
375 (Fig. 5c). Levels of donor chimerism were stable thereafter, and no signs of GVHD or
376 immune rersponses to the GFP neo-antigen were detected in animals observed longitudi-
377 nally.

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

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