Manipulating immune tolerance with micro-RNA regulated gene therapy

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The success of *in vivo* gene therapy greatly depends on the ability to control the immune response toward the therapeutic transgene. Over the last decade several vector-based and pharmacological approaches have been explored to control the immune-mediated clearance of transgene-expressing cells after viral delivery. One important outcome from these studies is the concept that expression of a transgene in tolerance-promoting organs, such as the liver and tolerogenic antigen-presenting cells, can help safeguard transgene-expressing cells from immune-mediated clearance. Gene therapists are now manipulating vectors to target naturally occurring tolerogenic properties of the body by: (i) incorporating tissue/cell specific promoters for targeted expression, (ii) using viral-capsid engineering to alter tropism and avoid pre-existing immunity, and (iii) regulating cell and activation dependent expression by including micro-RNA (miR) targets into expression cassettes. The combination of these three layers of vector regulation greatly enhances the targeting of tolerogenic cells and limits off-target expression of the transgene, which can lead to the induction of transgene-specific pathogenic effector T cells. In this review, we discuss the application of using miR transgene regulation to generate tolerogenic responses and speculate on possible mechanisms used by the liver to induce the transgene-specific regulatory T cells.

Keywords: Treg, micro-RNA, gene therapy, hepatocytes, tolerance, transgene, regulatory cells, liver

INTRODUCTION

A major obstacle for achieving therapeutic efficacy when using *in vivo* gene therapy is the development of an immune response toward the transgene since pathogenic immune responses can lead to the clearance of transgene-expressing cells. While long-term transgene expression has been achieved in several clinical trials using direct viral-vector administration (Kaplitt et al., 2007; Bainbridge et al., 2008; Hauswirth et al., 2008), targeting immune-privileged sites like the eye, brain, and testis as was done in these trials suggests that selective transgene expression in immune-privileged tissues (for review see Lowenstein et al., 2007) may diminish but not necessarily abolish the need for vector modification and/or pharmacological help to maintain transgene expression. Unfortunately for many individuals in need of gene therapy, disease correction requires delivery of a vector to immune-competent organs and requires robust local or systemic expression of a therapeutic protein. To combat the potential loss of transgene expression due to immune complications, gene therapists are modifying gene delivery platforms and using therapeutic regimens (such as immunosuppressive drugs) to promote tolerance toward the transgene. In this review, we focus on the use of vector modification with micro-RNA (miR) targets to regulate transgene expression and how they can be used with other layers of vector modification to promote transgene tolerance.

IMMUNE TOLERANCE

Tolerance is a naturally occurring process that uses one or more immune mechanisms to achieve immunological homeostasis. One means to achieve a tolerogenic state toward an antigen (Ag) is through passive tolerance, which is a quiescent immunological state where T cells do not come into contact with their cognate Ag or where effector T cells (Teff) are deleted or anergized upon Ag recognition, resulting in immune ignorance. Active tolerance, on the other hand, is a dynamic process whereby FoxP3-expressing regulatory T cells (Treg) work to suppress inflammatory immune responses in Ag-specific and non-specific ways (Fehervari and Sakaguchi, 2004a; Sakaguchi et al., 2008). Among the Treg sub-populations are the naturally occurring Treg (nTreg), which are differentiated in the thymus, and the induced Treg (iTreg) which are induced in the periphery, are phenotypically similar and have similar regulatory properties; however, the relative contribution of iTreg and nTreg in regulating immune responses in different disease states is subject to ongoing discussions. Nevertheless, Treg induction and expansion have become a focal point by some gene therapists for developing tolerance to therapeutic proteins because of their potent regulatory properties (Luth et al., 2008; Nayak et al., 2009).

TOLEROGENIC NATURE OF THE LIVER

Liver architecture creates a unique immunological site where circulating Ags and immune cells can meet. In a healthy state, natural tolerogenic mechanisms of the liver ensure that immunity against innocuous Ag (gut-derived nutrients and damaged/aged cells) and potentially immunogenic Ag (gut flora-derived) are kept in check. The means by which the liver induces this tolerance is partly attributed to the composition and diversity of antigen-presenting

cells (APC) and the presence of regulatory cytokines. The liver accommodates a variety of cells equipped to engage T cells, including canonical APC like liver-resident macrophages (Kupffer cells, KC) and conventional- and plasmacytoid-dendritic cells (cDC, pDC respectively), and non-canonical APC like liver sinusoidal endothelial cells (LSEC), and stellate cells (SC) and hepatocytes that are able to process and present Ag, secrete regulatory cytokines, and provide co-stimulatory molecules (Knolle et al., 1999; Limmer et al., 2000; Warren et al., 2006). Furthermore, all of these cells, with the exception of hepatocytes [which are deficient in MHC-class (MHC) – II expression] have been implicated in the generation of Treg and reported as one of the important biomarkers for predicting successful liver engraftment without immunosuppression (Seyfert-Margolis and Turka, 2008).

The tolerogenic properties of liver APC can be ascribed to the consistent expression of anti-inflammatory cytokines and other tolerogenic molecules found in a hepatic micro-environment. The liver is a rich source of IL-10 and TGF-β, both of which are potent modulators of APC differentiation, trafficking, and function (Lau and Thomson, 2003). In the absence of the IL-10 gene or blocking IL-10 with a recombinant IL-10R fusion protein, hepatic tolerance is not established in a ConA induced model of hepatitis, signifying the central role of this cytokine in maintaining tolerance (Erhardt et al., 2007). In the Erhardt et al. study, KC were shown to be the key source of IL-10, but more importantly, the lack of IL-10 expression by Treg was a critical factor for resolving inflammation, suggesting that IL-10 is needed by APC and T cells to maintain tolerance. TGF-β was originally recognized as a growth factor for non-immune cells and only later as an inhibitory signal for activation of immune cells, preventing activation of macrophages and maturation of DC and modulating effector functions of T and B cells (Chen and Konkel, 2010). Chen and colleagues showed that TGF-β signaling coupled with TCR stimulation convert $CD4^+CD25^-Foxp3^-$ into iTreg. Thus, hepatic architecture, position, and micro-environment encourage interactions between non-parenchymal cells and circulating lymphocytes in the presence of immunoregulatory cytokines. Additionally, 50% of hepatic Treg are also $Ki-67^+$ (unpublished data by our group) supporting the hypothesis that the liver could be a preferential site for the expansion of Treg. Overall, these features distinguish the liver as a potential site for the development and expansion of acquired peripheral tolerance.

PROMOTER AND VECTOR MODULATION TO REDUCE TRANSGENE IMMUNITY

Tissue-specific promoters that restrict transgene expression to a targeted cell type have been widely employed in gene therapy to avoid immune responses. The basic concept for using tissue-specific promoters is to drive expression of the therapeutic transgene to target cells while preventing its expression in APC in order to avoid the priming of Teff (Vandendriessche et al., 2007). Despite the specificity of tissue-specific promoters, their use as a regulatory layer does not necessarily prevent immune responses toward the encoded transgene. We among others have found that the use of promoters specific for tolerogenic cells such as hepatocytes can still lead to transgene-specific neutralizing antibodies and

immune-mediated clearance of transgene-expressing cells (Brown et al., 2004; Follenzi et al., 2007; Herzog, 2007; Haribhai et al., 2011; Mingozzi and High, 2011). These results may be due to non-specific activity of the promoter, cross-priming, or in the case of integrating vectors, promoter-enhancer trapping at insertions near active regions of transcription (De Palma et al., 2005). Taken together, tissue-specific promoters can foster transgene stability by avoiding immune responses and enhancing tissue targeting in many cases; however, the use of tissue-specific promoters does not guarantee that transgene tolerance will be achieved in all immunological settings.

Alternating viral-capsid proteins to avoid pre-existing immunity and/or the development of immunity toward the vector and transgene is another approach by which gene therapists can maximize transgene expression. Viral capsids are immunogens, which can trigger innate and adaptive immune responses and lead to the development of neutralizing antibodies toward the vector as well as capsid-specific-Teff responses. In fact, it is believed that the first clinical trial for hemophilia B using adeno-associated vector (AAV) to deliver the factor IX (FIX) transgene failed due to pre-existing anti-AAV immune responses that led to the clearance of the transduced cells (Prasad et al., 2001; Li et al., 2007; Mingozzi et al., 2007). The use of pseudocapsids or particular serotypes with viral gene therapy can avoid existing antibody or cellular immune responses to the vector if the Ags of the introduced capsid protein(s) are naïve/less immunogenic to the immune system. In addition, swapping or novel capsid proteins can alter tropism of the vector and de-target APC. Kang et al. (2005) showed that in the case of long-term factor VIII (FVIII) expression the use of a gp64 capsid protein in lieu of the canonical VSV-G capsid protein in a feline immunodeficiency vector was able to partially correct disease in hemophilia A mice. However, when a gp64 lentiviral (LV) vector was used in the same mouse model, Lillicrap and colleagues were unable to achieve long-term FVIII expression unless the expression was combined with another layer of gene regulation (Matsui et al., 2011). While these results suggest that tissue-specific promoters and viral pseudotyping can improve transgene expression and long-term outcome of gene therapy with viral vectors, they also suggest that more regulation may be required to achieve transgene-specific tolerance in more stringent immunological settings.

miR TRANSGENE REGULATION

Molecular engineering of viral-vector cassettes with miR targets is the latest attempt at regulating expression in gene therapy. miR are a family of endogenously expressed, small, non-coding RNA of ∼22 nucleotides that are post-transcriptional regulators of gene expression and inhibit expression by binding to miR targets on mRNA to repress transcription or to promote mRNA degradation. Similar to the way miR regulates the expression of genes naturally encoding miR targets, vectors engineered with miR targets can prevent transgene expression in the transduced cells provided the target cells express the particular miR matching the miR target encoded in the vector (Brown and Naldini, 2009). Since miR are intimately involved in a variety of immunological processes, including B and T cell development, hematopoietic cell differentiation, apoptosis, and proliferation (Landgraf et al., 2007; Kelly

and Russell, 2009), the incorporation of miR target sequences in vectors makes it possible to direct expression to or away from particular cells of the immune system by exploiting their natural miR profile. miR expression profiles vary considerably from being constitutively expressed or induced to shut off, depending on cell type and maturation state (Petriv et al., 2010). This variability suggests that by incorporating miR targets in vectors, it is possible to target transgene expression not only to certain cell types, but also to certain stages of cell differentiation or activation. The ability to regulate gene expression at a state-specific level is of particular interest to gene therapists because it adds a new dimension of regulating gene expression that cannot be achieved by other vector modifications.

miR TO GENERATE IMMUNE TOLERANCE

One attractive approach to generate immune tolerance is to direct transgene expression toward APC in a tolerogenic state. The immature state of some APC is considered to have tolerogenic properties because they lack the necessary molecules needed to induce robust immune responses. Our group among others has shown that immature dendritic cells (iDC) are a potent inducer of Treg and, therefore, have become targets for inducing Treg (Wakkach et al., 2003; Levings et al., 2005; Yamazaki and Steinman, 2009). The mechanisms by which iDC promote Treg induction is through the expression of tolerance-promoting cytokines, such as IL-10 (Gregori et al., 2010) and TGF-β (Wakkach et al., 2003; Fehervari and Sakaguchi, 2004b) and the surface expression of tolerogenic co-stimulatory molecules such as PDL-1 (Unger et al., 2009); however, a major caveat for targeting iDC is the short window of opportunity for maximizing their tolerogenic abilities since activated DC produce inflammatory cytokines and up-regulate co-stimulatory molecules that promote the development of Teff. Therefore, in the scenario of exploiting the tolerogenic state of APC it is vital to limit Ag expression to the tolerogenic state of the cell and turning off expression once the APC is activated. Using miR targets in the vector can take advantage of the unique state-specific miR expression of APC to accomplish this goal. iDC, for example, do not express miR-122, -146a, or -155 in the immature state, however, upon activation by cytokines or TLR ligands these miR are induced and become highly expressed by the DC (Taganov et al., 2006; Jurkin et al., 2010). Therefore, using a vector regulated by miR targets encoding for miR-122, -146a, or -155 targets could eliminate transgene expression of transgene in DC upon activation. Brown et al. (2007b) showed that state-specific regulation is possible with a LV.GFP miR-155 target encoded construct, where GFP is selectively expressed in the immature state of human peripheral blood-derived DC, while GFP expression was dramatically reduced upon LPS activation of the DC. While this approach has been proven feasible for selective targeting of iDC, more work needs to be done to determine the immunological consequence of this approach and whether it can be used *in vivo*.

An established strategy for generating transgene tolerance with miR-containing vectors is to de-target transgene expression of the hematopoietic system while expressing the transgene in hepatocytes. All cells of the hematopoietic system highly express miR-142; therefore, vectors including miR-142 targets can repress transgene

expression in all cells of this lineage including APC. The effectiveness of using miR-142-targeted vectors to eliminate expression in APC and generate transgene tolerance was first achieved by Naldini and colleagues who showed that including miR-142 target repeats in a LV.GFP vector reduced GFP expression in mouse macrophages and DC to undetectable levels while maintaining GFP expression in non-hematopoietic tissues (Brown et al., 2006). The same group went on to show that hemophilia B can be cured when FIX deficient mice receive a LV expressing human FIX under the regulation of miR-142 targets, whereas the animals receiving the same LV without miR-142 targets cleared the transgene-expressing cells (Brown et al., 2007a). Interestingly, the original belief that miR-142-targeted vectors promote transgene tolerance through immune ignorance due to de-targeting of APC recently changed when we showed induction of Treg using a LV. GFP, miR-142-targeted vectors with liver-specific promoters mediate transgene tolerance (Annoni et al., 2009). To prove that active tolerance is at play in regulating transgene expression with miR-142 vectors, we showed that in a collaborative effort that mice injected with LV encoding the Ag ovalbumin (OVA), liver-specific promoter, and miR-142-targeted sequences along with naïve, OVA-specific T cells induces OVA-specific Treg, suggesting that Ag-specific iTreg are regulating transgene tolerance (Matrai et al., 2011). The concept that Ag-specific Treg are regulating tolerance is supported by the fact that upon pDNA re-challenge with the transgene, tolerized mice showed little response to the Ag, while mice that cleared the transgene-expressing cells showed increased reactivity (Annoni et al., 2009; Matrai et al., 2011).

An important point to consider in most of the experimental conditions used to generate transgene tolerance with miR targets is the preferential targeting of the transgene to the hepatocytes. It is important to note, however, that targeting expression to hepatocytes and de-tartgeting APC do not guarantee transgene persistence. Osborn et al. (2011) showed in a mucopolysaccharidosis type I mouse model that hydrodynamic injection of minicircle DNA expressing α-L-iduronidase (IDUA) regulated by liver-specific promoter and miR-142 targets mice do not tolerate IDUA and must receive immunosuppression in order to achieve long-term transgene expression. In this study it was unclear why IDUA expression was lost; however, it was speculated that immunogenicity of the transgene and/or the acute inflammation from the hydrodynamic injection lead to the clearance of the transgene. This result stresses the importance of selecting a vector delivery platform/method that has the least amount of immunogenicity and selecting or modifying the transgene in order to reduce the immune response. Despite the failure of this protocol, targeting hepatocytes still remains one of the best options for gene delivery because of their ability to secrete high levels of protein and the innate tolerogenic properties; however, the exact mechanism(s) the liver uses to induce tolerance still remains a mystery. To better understand how hepatocyte-targeted gene therapy with miR regulated vectors generates transgene-specific Treg after gene therapy we are addressing the tolerogenic properties particular to the liver-resident cells that promote tolerogenic responses.

HOW TARGETING AG EXPRESSION TO HEPATOCYTES BY MIR-REGULATED VECTOR GENERATES TOLERANCE: A POSSIBLE MECHANISM

Based on our data, we have developed a hypothesis explaining the mechanisms leading to tolerance using liver-directed, miR-142 regulated LV gene therapy. So far, we have learned that LV containing miR-142 targets (LV.142T) and conventional LV have similar transduction efficiency and immunogenicity toward vector components. In fact, the immune response to LV.142T vector RNA compared to conventional LV vectors in infected pDC results in comparable levels of type 1 IFN release after TLR7 triggering (Brown et al., 2007c). However, since LV.142T suppresses transgene expression in APC, the encoded Ag avoids presentation by IFN α-β-activated professional APC, thus circumventing the induction of a robust transgene-specific CD8$^+$ Teff cell responses. Nevertheless, CD8$^+$ T cell priming occurs among LV.142T infected hepatocytes due to their expression of MHC-I molecule and co-stimulatory molecules; however, Teff priming by hepatocytes is short-lived due to the low levels of MHC-I expression and lack of key co-stimulatory molecules needed to sustain Teff activation and expansion. Therefore, in this scenario miR-142 regulation indirectly causes alterations to T cell signal 1 (MHC-pep) and signal 2 (co-stimulation) that are necessary for optimal T cell priming. In addition, very little is known about signal 3 (cytokine milieu) at the time of Ag-presentation in this model, but given the reduced priming and Teff memory formation using LV.142T, it is likely that altered levels of IL-7, IL-15, and TGF-β in the liver micro-environment play a role in reducing the Teff response (Sawa et al., 2009). Thus, despite de-targeting APC with LV.142T, transgene-specific CD8$^+$ Teff do occur, but since they are suboptimally primed by the hepatocytes, there is a premature contraction phase and reduced cytotoxic effects.

It is difficult to interpret how the generation of Treg occurs using LV.142T vectors since the primary expressing cell (hepatocytes) inefficiently express MHC-II necessary for priming CD4$^+$ T cells, and the transgene is not expressed in the professional APC. Our hypothesis is that CD4$^+$ T cells are primed by APC that have engulfed the Ag expelled from transduced dead or dying hepatocytes and/or by engulfing the dead cells and cross-presenting the Ag. After engulfment and presentation by APC, the Ag can prime naïve CD4$^+$ T cells under a tolerogenic cytokine milieu (signal 3) that promotes the induction of transgene-specific Treg cells. Since the liver micro-environment is rich in TGF-β which is required to convert naïve CD4$^+$ T cells into iTreg (Chen et al., 2003), it is likely the low level of Ag-presentation by APC combined with the presence of TGF-β provided by the hepatocytes that evokes iTreg generation. In addition, we have demonstrated that active tolerance to transgene expressed via integrase defective (ID) IDLV.142T platform outlasts the window of transgene expression (Matrai et al., 2011), suggesting that tolerance induction and Tregs conversion are related to tolerogenic Ag-presentation rather than Ag-persistence.

CONCLUSION

In summary, transgene immunity can be overcome when using *in vivo* gene therapy by exploiting vectors that have been designed to take advantage of the tolerogenic properties of the body. The recent incorporation of molecular de-targeting techniques achieved with miR targets combined with tissue-specific promoters and capsid engineering provides a potent regulatory scheme that, when designed correctly, can induce Treg capable of controlling transgene-specific-Teff and prolong transgene expression. While miR targeting of viral vectors has not been tested in humans, the transition of miR targets into clinical vectors is very appealing since most clinical trials will be challenged by the problem of maintaining immune tolerance. However, before miR targeted vectors can be widely accepted, they must be tested in large-animal models for efficacy, safety and confirmation that the approach of targeting the liver to induce tolerance is similar in rodents and non-human primates. In due time, it is likely that miR regulation will become commonplace in the clinical arena given its efficiency, potential tolerogenic properties, and the broad spectrum of miR that are naturally expressed.

REFERENCES

Annoni, A., Brown, B. D., Cantore, A., Sergi, L. S., Naldini, L., and Roncarolo, M. G. (2009). In vivo delivery of a microRNA-regulated transgene induces antigen-specific regulatory T cells and promotes immunologic tolerance. *Blood* 114, 5152–5161.

Bainbridge, J. W., Smith, A. J., Barker, S. S., Robbie, S., Henderson, R., Balaggan, K., Viswanathan, A., Holder, G. E., Stockman, A., Tyler, N., Petersen-Jones, S., Bhattacharya, S. S., Thrasher, A. J., Fitzke, F. W., Carter, B. J., Rubin, G. S., Moore, A. T., and Ali, R. R. (2008). Effect of gene therapy on visual function in Leber's congenital amaurosis. *N. Engl. J. Med.* 358, 2231–2239.

Brown, B. D., Cantore, A., Annoni, A., Sergi, L. S., Lombardo, A., Della Valle, P., D'angelo, A., and Naldini, L. (2007a). A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood* 110, 4144–4152.

Brown, B. D., Gentner, B., Cantore, A., Colleoni, S., Amendola, M., Zingale, A., Baccarini, A., Lazzari, G., Galli, C., and Naldini, L. (2007b). Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat. Biotechnol.* 25, 1457–1467.

Brown, B. D., Sitia, G., Annoni, A., Hauben, E., Sergi, L. S., Zingale, A., Roncarolo, M. G., Guidotti, L. G., and Naldini, L. (2007c). In vivo administration of lentiviral vectors triggers a type I interferon response that restricts hepatocyte gene transfer and promotes vector clearance. *Blood* 109, 2797–2805.

Brown, B. D., and Naldini, L. (2009). Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications. *Nat. Rev. Genet.* 10, 578–585.

Brown, B. D., Shi, C. X., Rawle, F. E., Tinlin, S., Mckinven, A., Hough, C., Graham, F. L., and Lillicrap, D. (2004). Factors influencing therapeutic efficacy and the host immune response to helper-dependent adenoviral gene therapy in hemophilia A mice. *J. Thromb. Haemost.* 2, 111–118.

Brown, B. D., Venneri, M. A., Zingale, A., Sergi Sergi, L., and Naldini, L. (2006). Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat. Med.* 12, 585–591.

Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., Mcgrady, G., and Wahl, S. M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* 198, 1875–1886.

Chen, W., and Konkel, J. E. (2010). TGF-beta and 'adaptive' Foxp3(+) regulatory T cells. *J. Mol. Cell Biol.* 2, 30–36.

De Palma, M., Montini, E., Santoni De Sio, F. R., Benedicenti, F., Gentile, A., Medico, E., and Naldini, L. (2005). Promoter trapping reveals significant differences in integration site selection between MLV and HIV vectors in primary hematopoietic cells. *Blood* 105, 2307–2315.

Erhardt, A., Biburger, M., Papadopoulos, T., and Tiegs, G. (2007). IL-10, regulatory T cells, and Kupffer cells mediate tolerance in concanavalin A-induced liver injury in mice. *Hepatology* 45, 475–485.

Fehervari, Z., and Sakaguchi, S. (2004a). CD4+ Tregs and immune control. *J. Clin. Invest.* 114, 1209–1217.

Fehervari, Z., and Sakaguchi, S. (2004b). Control of Foxp3+ CD25+CD4+ regulatory cell activation and function by dendritic cells. *Int. Immunol.* 16, 1769–1780.

Follenzi, A., Santambrogio, L., and Annoni, A. (2007). Immune responses to lentiviral vectors. *Curr. Gene Ther.* 7, 306–315.

Gregori, S., Tomasoni, D., Pacciani, V., Scirpoli, M., Battaglia, M., Magnani, C. F., Hauben, E., and Roncarolo, M. G. (2010). Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* 116, 935–944.

Haribhai, D., Williams, J. B., Jia, S., Nickerson, D., Schmitt, E. G., Edwards, B., Ziegelbauer, J., Yassai, M., Li, S. H., Relland, L. M., Wise, P. M., Chen, A., Zheng, Y. Q., Simpson, P. M., Gorski, J., Salzman, N. H., Hessner, M. J., Chatila, T. A., and Williams, C. B. (2011). A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity* 35, 109–122.

Hauswirth, W. W., Aleman, T. S., Kaushal, S., Cideciyan, A. V., Schwartz, S. B., Wang, L., Conlon, T. J., Boye, S. L., Flotte, T. R., Byrne, B. J., and Jacobson, S. G. (2008). Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. *Hum. Gene Ther.* 19, 979–990.

Herzog, R. W. (2007). Immune responses to AAV capsid: are mice not humans after all? *Mol. Ther.* 15, 649–650.

Jurkin, J., Schichl, Y. M., Koeffel, R., Bauer, T., Richter, S., Konradi, S., Gesslbauer, B., and Strobl, H. (2010). miR-146a is differentially expressed by myeloid dendritic cell subsets and desensitizes cells to TLR2-dependent activation. *J. Immunol.* 184, 4955–4965.

Kang, Y., Xie, L., Tran, D. T., Stein, C. S., Hickey, M., Davidson, B. L., and Mccray, P. B. Jr. (2005). Persistent expression of factor VIII in vivo following non primate lentiviral gene transfer. *Blood* 106, 1552–1558.

Kaplitt, M. G., Feigin, A., Tang, C., Fitzsimons, H. L., Mattis, P., Lawlor, P. A., Bland, R. J., Young, D., Strybing, K., Eidelberg, D., and During, M. J. (2007). Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. *Lancet* 369, 2097–2105.

Kelly, E. J., and Russell, S. J. (2009). MicroRNAs and the regulation of vector tropism. *Mol. Ther.* 17, 409–416.

Knolle, P. A., Germann, T., Treichel, U., Uhrig, A., Schmitt, E., Hegenbarth, S., Lohse, A. W., and Gerken, G. (1999). Endotoxin down-regulates T cell activation by antigen-presenting liver sinusoidal endothelial cells. *J. Immunol.* 162, 1401–1407.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A. O., Landthaler, M., Lin, C., Socci, N. D., Hermida, L., Fulci, V., Chiaretti, S., Foa, R., Schliwka, J., Fuchs, U., Novosel, A., Muller, R. U., Schermer, B., Bissels, U., Inman, J., Phan, Q., Chien, M., Weir, D. B., Choksi, R., De Vita, G., Frezzetti, D., Trompeter, H. I., Hornung, V., Teng, G., Hartmann, R., Palkovits, M., Di Lauro, R., Wernet, P., Macino, G., Rogler, C. E., Nagle, J. W., Ju, J., Papavasiliou, F. N., Benzing, T., Lichter, P., Tam, W., Brownstein, M. J., Bosio, A., Borkhardt, A., Russo, J. J., Sander, C., Zavolan, M., and Tuschl, T. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 129, 1401–1414.

Lau, A. H., and Thomson, A. W. (2003). Dendritic cells and immune regulation in the liver. *Gut* 52, 307–314.

Levings, M. K., Gregori, S., Tresoldi, E., Cazzaniga, S., Bonini, C., and Roncarolo, M. G. (2005). Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood* 105, 1162–1169.

Li, C., Hirsch, M., Asokan, A., Zeithaml, B., Ma, H., Kafri, T., and Samulski, R. J. (2007). Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid in vivo. *J. Virol.* 81, 7540–7547.

Limmer, A., Ohl, J., Kurts, C., Ljunggren, H. G., Reiss, Y., Groettrup, M., Momburg, F., Arnold, B., and Knolle, P. A. (2000). Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat. Med.* 6, 1348–1354.

Lowenstein, P. R., Mandel, R. J., Xiong, W. D., Kroeger, K., and Castro, M. G. (2007). Immune responses to adenovirus and adeno-associated vectors used for gene therapy of brain diseases: the role of immunological synapses in understanding the cell biology of neuroimmune interactions. *Curr. Gene Ther.* 7, 347–360.

Luth, S., Huber, S., Schramm, C., Buch, T., Zander, S., Stadelmann, C., Bruck, W., Wraith, D. C., Herkel, J., and Lohse, A. W. (2008). Ectopic expression of neural autoantigen in mouse liver suppresses experimental autoimmune neuroinflammation by inducing antigen-specific Tregs. *J. Clin. Invest.* 118, 3403–3410.

Matrai, J., Cantore, A., Bartholomae, C. C., Annoni, A., Wang, W., Acosta-Sanchez, A., Samara-Kuko, E., De Waele, L., Ma, L., Genovese, P., Damo, M., Arens, A., Goudy, K., Nichols, T. C., Von Kalle, C., Mk, L. C., Roncarolo, M. G., Schmidt, M., Vandendriessche, T., and Naldini, L. (2011). Hepatocyte-targeted expression by integrase-defective lentiviral vectors induces antigen-specific tolerance in mice with low genotoxic risk. *Hepatology* 53, 1696–1707.

Matsui, H., Hegadorn, C., Ozelo, M., Burnett, E., Tuttle, A., Labelle, A., Mccray, P. B. Jr., Naldini, L., Brown, B., Hough, C., and Lillicrap, D. (2011). A microRNA-regulated and GP64-pseudotyped lentiviral vector mediates stable expression of FVIII in a murine model of Hemophilia A. *Mol. Ther.* 19, 723–730.

Mingozzi, F., and High, K. A. (2011). Immune Responses to AAV in Clinical Trials. *Curr. Gene Ther.* 11, 321–330.

Mingozzi, F., Maus, M. V., Hui, D. J., Sabatino, D. E., Murphy, S. L., Rasko, J. E., Ragni, M. V., Manno, C. S., Sommer, J., Jiang, H., Pierce, G. F., Ertl, H. C., and High, K. A. (2007). CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat. Med.* 13, 419–422.

Nayak, S., Cao, O., Hoffman, B. E., Cooper, M., Zhou, S., Atkinson, M. A., and Herzog, R. W. (2009). Prophylactic immune tolerance induced by changing the ratio of antigen-specific effector to regulatory T cells. *J. Thromb. Haemost.* 7, 1523–1532.

Osborn, M. J., Mcelmurry, R. T., Lees, C. J., Defeo, A. P., Chen, Z. Y., Kay, M. A., Naldini, L., Freeman, G., Tolar, J., and Blazar, B. R. (2011). Minicircle DNA-based gene therapy coupled with immune modulation permits long-term expression of alpha-L-iduronidase in mice with mucopolysaccharidosis type I. *Mol. Ther.* 19, 450–460.

Petriv, O. I., Kuchenbauer, F., Delaney, A. D., Lecault, V., White, A., Kent, D., Marmolejo, L., Heuser, M., Berg, T., Copley, M., Ruschmann, J., Sekulovic, S., Benz, C., Kuroda, E., Ho, V., Antignano, F., Halim, T., Giambra, V., Krystal, G., Takei, C.

J., Weng, A. P., Piret, J., Eaves, C., Marra, M. A., Humphries, R. K., and Hansen, C. L. (2010). Comprehensive microRNA expression profiling of the hematopoietic hierarchy. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15443–15448.

Prasad, S. A., Norbury, C. C., Chen, W., Bennink, J. R., and Yewdell, J. W. (2001). Cutting edge: recombinant adenoviruses induce CD8 T cell responses to an inserted protein whose expression is limited to non immune cells. *J. Immunol.* 166, 4809–4812.

Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008). Regulatory T cells and immune tolerance. *Cell* 133, 775–787.

Sawa, Y., Arima, Y., Ogura, H., Kitabayashi, C., Jiang, J. J., Fukushima, T., Kamimura, D., Hirano, T., and Murakami, M. (2009). Hepatic interleukin-7 expression regulates T cell responses. *Immunity* 30, 447–457.

Seyfert-Margolis, V., and Turka, L. A. (2008). Marking a path to transplant tolerance. *J. Clin. Invest.* 118, 2684–2686.

Taganov, K. D., Boldin, M. P., Chang, K. J., and Baltimore, D. (2006). NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12481–12486.

Unger, W. W., Laban, S., Kleijwegt, F. S., Van Der Slik, A. R., and Roep, B. O. (2009). Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur. J. Immunol.* 39, 3147–3159.

Vandendriessche, T., Thorrez, L., Acosta-Sanchez, A., Petrus, I., Wang, L., Ma, L., L, D. E. W., Iwasaki, Y., Gillijns, V., Wilson, J. M., Collen, D., and Chuah, M. K. (2007). Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 vs. lentiviral vectors for hemophilia B gene therapy. *J. Thromb. Haemost.* 5, 16–24.

Wakkach, A., Fournier, N., Brun, V., Breittmayer, J. P., Cottrez, F., and Groux, H. (2003). Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 18, 605–617.

Warren, A., Le Couteur, D. G., Fraser, R., Bowen, D. G., Mccaughan, G. W., and Bertolino, P. (2006). T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells. *Hepatology* 44, 1182–1190.

Yamazaki, S., and Steinman, R. M. (2009). Dendritic cells as controllers of antigen-specific Foxp3+ regulatory T cells. *J. Dermatol. Sci.* 54, 69–75.