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Commentary

LSpECifying transgene expression

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The ultimate goal of liver gene therapy for monogenic diseases is to provide safe, therapeutic, life-long replacement of the missing function to affected patients by a single administration. Avoiding immunity and achieving immune tolerance to the transgene-encoded protein is of fundamental importance to reach this goal. Adeno-associated viral (AAV) vectors delivering a functional coagulation factor VIII (FVIII) or IX (FIX) transgene have shown multi-year therapeutic benefit in adults with hemophilia, following a single intravenous administration. These have now become commercially available gene therapies, indicated for adult patients affected by hemophilia A or B, bleeding diseases due to deficiency of FVIII or FIX, respectively.[1,2] Importantly, treated patients did not develop anti-FVIII or anti-FIX antibodies (Abs). These data confirm many years of pre-clinical research supporting that AAV-based liver gene transfer is in most cases pro-tolerogenic rather than immunogenic, i.e., favors the induction of immune tolerance rather than immunity toward transgene-derived antigens (Ags).[3] Mingozzi et al. showed that AAV-mediated expression by hepatocytes induced FIX-specific regulatory T cells (Tregs) and immune tolerance *in vivo* in mice.[4] Similarly, lentiviral vector (LV)-based liver gene transfer was also associated to immune unresponsiveness and active immune tolerance toward transgene Ags.[5,6] Interestingly, the pro-tolerogenic outcome was promoted when expression of the transgene was made strictly hepatocyte specific by a combination of transcriptional and post-transcriptional microRNA-mediated control.[7] In this construct, target sequences of the hematopoietic-cell-specific microRNA 142, in the transgene 3′ UTR, mediate transgene RNA degradation in antigen-presenting cells, thus allowing for cell-specific de-targeting of transgene expression.[7,8] The liver pro-tolerogenic properties are well known for decades because of the successful transplantation of allogeneic livers[9] and the induction of immune tolerance toward portal vein-delivered Ags.[10] The exact mechanisms by which the liver can skew the immune system toward tolerance remain to be fully understood. Different cell types have been implicated in this process, among which are hepatocyte themselves and liver sinusoidal endothelial cells[11] (LSECs).

In this issue of *Molecular Therapy Nucleic Acids*, Borsotti et al. describe the use of an LSEC-specific promoter based on the endogenous stabilin-2 (STAB2) promoter inside LVs, driving expression of GFP as marker gene, or FVIII as therapeutic transgene for hemophilia A. Contrary to a ubiquitously expressed GFP, LSEC-specific GFP expression is maintained long term in LV-treated mice, indicating lack of anti-GFP immune responses. This is confirmed even if anti-GFP clonal cytotoxic T cells (Jedi)[12] are adoptively transferred to LV.STAB2-GFP-treated mice, suggesting active immune tolerance toward the GFP. When FVIII transgenes are expressed by LVs with the STAB2 promoter, partial therapeutic activity (10%–15% of normal) is maintained stable and long term, in the absence of anti-FVIII immunity. If Tregs are depleted, a transient rise in anti-FVIII Abs is observed. This work builds upon previous achievements of the Follenzi group, following the concept that expressing transgenes in endothelial cells can induce immunological tolerance. This article confirms and expands previous findings reported by the same group using different endothelial promoters, based on VE-cadherin (VEC) and endogenous FVIII promoter.[13,14]

LV.STAB2 represents the evolution of the LV.VEC platform, by further restricting transgene expression to LSECs among the endothelial cells. LSEC-specific transgene expression amplified the tolerogenic capacity of LV.VEC delivery, as shown by persistence of GFP-expressing LSECs in mice after adoptive transfer of the anti-GFP Jedi CD8 T cells. Conversely, GFP expression was eradicated by Jedi CD8 T cells in LV.VEC-treated mice.[13] Phenotypic analysis of immune cells in the liver of LV.STAB2-treated mice revealed an abundant recruitment of CD8 T cells, which showed a significant upregulation of PD1 compared with those in untreated mice. PD1 expression was not associated with a specific LV construct or with the transfer of Jedi CD8 T cells. Therefore, the PD1-PDL1 pathway does not seem to play a key role in the induction of tolerance to the transgene in LV.STAB2-treated mice, rather may represent a naturally occurring regulatory loop to control excessive T cell activation. However, tolerance in LV.STAB2-treated mice was associated with a reduced killing activity of CD8 T cells *in vivo* and *in vitro*, thus suggesting a permanent inactivation ascribable to an exhaustion state, which remains an interesting aspect to be elucidated.

Application of LV.STAB2 platform for FVIII gene addition confirmed previous achievements. Stable FVIII expression was established *in vivo* in adult hemophilia A mice in absence of humoral and cellular immune responses, as obtained using the VEC promoter or the endogenous FVIII promoter.[13,14] An advantage of LV-mediated compared with AAV-mediated liver gene transfer is the potential to be maintained upon target cell proliferation, such as during liver growth and homeostatic turnover. For this reason, it would be useful to monitor the stability of *in vivo* gene transfer to LSECs following LV delivery to newborn mice. In terms of immunological characterization, in this work, Borsotti and colleagues show that Treg depletion temporary re-awake

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anti-FVIII immune responses, without any loss of FVIII-expressing LSECs. This result is intriguing and the underlying mechanisms are worth further investigation. Indeed, in absence of Tregs, only CD4 T and B lymphocytes were free to react toward FVIII, respectively, providing "helper" function to anti-FVIII Abs production. Conversely, CD8 T lymphocytes did not re-acquire their killing capacity, thus explaining the recovery of FVIII activity to pre-Treg depletion levels, without any sign of immune-mediated loss of FVIII-expressing LSECs.

LVs are emerging as versatile tools for *in vivo* gene delivery to the liver, as they can be made alloantigen free and phagocytosis shielded,[15,16] thus potentially improving the efficiency of gene transfer to LSECs, as well as to hepatocytes, and transgene expression can be effectively tailored to specific cell types. Many elements of the mechanisms of immune response to LV-encoded Ags in the liver appear to converge whether instructing transgene expression to LSECs, by STAB2 promoter, or to hepatocytes, by the previously described cassette, composed by the enhanced transthyretin promoter and target sequences of microRNA 142.[6] Induction of transgene-specific Tregs was demonstrated when targeting to hepatocytes expression of GFP and ovalbumin as model Ags, or alternatively, FIX to correct hemophilia B or even an insulin immunodominant T cell epitope to prevent type 1 diabetes.[17–20] However, in all these cases the persistence of transgene in hepatocytes has been shown to be Treg dependent.[6,18,19] In addition, we reported that interferon-γ-dependent PDL1 upregulation by hepatocytes played a role in controlling the expansion of transgene-specific CD8 T cells, which did not display a permanent loss of killing activity, revealed in the absence of Treg *in vivo* and *in vitro*.[20]

Overall, these studies highlight the complexity of the immune responses triggered by *in vivo* LV gene transfer to the liver and suggest that deeper understanding and the capacity to control these mechanisms will offer a wide range of applications not only to treat genetic diseases but also to counteract immune-mediated disorders.

DECLARATION OF INTERESTS

A.A. and A.C. are inventors on patents and patent applications on LV technology related to the work described here.

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