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Successful RAG1-SCID gene therapy depends on the level of RAG1 expression

To the Editor:

An interesting recent publication by van Til et al¹ reports on attempts to correct murine recombination-activating gene 1 (*Rag1*) deficiency using lentiviral-mediated gene transfer. Using this approach, the authors were unable to fully correct the immune deficiency, yet oligoclonal T cells developed reminiscent of human Omenn syndrome, a disorder known to arise from hypomorphic *RAG* mutations, resulting in low recombinase activity.² Our previous work³ reported a much more successful restoration of *Rag1* deficiency using similar lentiviral vector technology. We obtained full restoration of peripheral T-cell numbers after 5 months using 2 different promoters (ubiquitous chromatin opening element [UCOE] and spleen focus-forming virus [SFFV]), approximately 35% of normal B-cell numbers, and importantly a polyclonal T-cell receptor (TCR) and B-cell receptor repertoire, allowing functional responses after immunization with the T-cell-dependent antigens. Altogether, much more successful functional restoration of both humoral and cellular adaptive immune responses is possible.

Because van Til et al used our lentiviral vectors, it is important to discuss these divergent results. As we⁴ have indicated before, the success of RAG1 gene therapy is highly dependent on the eventual *RAG1* expression in developing T and B cells. Gene expression profiling of human thymus, bone marrow, and murine immune tissues (www.immgen.org) demonstrate that the highest *RAG1* expression levels are found at the early double positive (DP) stages in thymic T-cell development.⁵ This coincides with the highest recombination activity of the TCR Alpha/Delta locus, which is the most complex TCR locus in mouse and human, containing more than 60 V α genes spanning a locus of more than 1.6 Mb. Indeed, in mice reconstituted with low *Rag1* expression, a block in development can be seen at the immature single positive to DP transition.

Therefore, the level of *RAG1* expression in transduced stem cells and after transplantation and reconstitution in lymphocytes is a crucial determinant of outcome. In our hands, we obtained expression levels of on average $0.7 \times$ ABL (Abelson murine leukemia viral oncogene homolog) per integrated copy at vector copy number of 3 (SFFV) and 0.6 (UCOE).³ Unfortunately, van Til et al¹ report very little data on *RAG1* expression in their samples,¹ but in Supplemental Fig 4, K and L, barely detectable *RAG1* expression in DN3 thymocytes is shown, whereas we observed on average $0.8 \times$ ABL expression (corresponding to

$\sim 30\%$ of wild-type *RAG1* expression in human thymus⁵). We therefore consider low expression of *RAG1* as the potential cause of the observed incomplete restoration of lymphocyte development. The reasons for this low *RAG1* expression can be several but might lie in different ways of producing lentiviral particles and a different unpublished transduction protocol. In addition to issues relating to *RAG1* expression levels, we emphasize that immunological and other nongenetic factors may play a role in the clinical phenotype of patients with RAG-severe combined immunodeficiency.⁶ Similarly, it is conceivable that conditions in animal facilities may also play a critical role in preclinical gene therapy models. This is underscored by the first successful attempts to correct *Rag1* deficiency using gamma-retroviral vectors,⁷ where mice with poor reconstitution never developed Omenn syndrome, thus indicating that conditions and sterility are important parameters of outcome.

These divergent results underscore the need for careful vector development, preclinical testing, and direct measurements of transgene expression. Although the results of van Til et al illustrate the complexity of gene therapy models, their results should be interpreted with caution and should not discourage scientists, clinicians, and regulators from efforts to cure RAG1-severe combined immunodeficiency through gene therapy approaches. The 2 studies collectively show that efficient gene transfer is a critical part of the protocol and is likely within reach in human cells using today's clinically approved protocols. To that end, we have developed novel vectors, with clinically acceptable promoters that are capable of high *RAG1* expression, and which are currently evaluated in preclinical work in mice and patient cells. This consorted international effort should lead up to a multicenter clinical trial for which funding has been secured.

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Reply

To the Editor:

In reply to the comment of Pike-Overzet and colleagues,¹ we are pleased with the opportunity to outline our position in the ongoing preclinical efficacy and safety evaluation of stem cell gene therapy in recombination-activating gene 1 deficient (*Rag1*^{-/-}) mice, in which we noted and analyzed a potential risk to develop inflammatory and autoimmune reactions resembling human Omenn syndrome, while maintaining low vector copy numbers (VCNs) per cell to minimize genotoxic risks. These results are seemingly divergent from an earlier publication by Pike-Overzet et al,² demonstrating reconstitution of T-cell functions; however, B-cell numbers remained reduced, especially with the elongation factor 1 α short (EFS) or ubiquitous chromatin opening element (UCOE) promoters, while T-cell receptor (TCR) rearrangements were not restored for all TCR isotypes, particularly in the UCOE promoter-treated mice. Pike-Overzet et al reached this partial correction of the murine *Rag1*^{-/-} phenotype with a significant failure rate: contrary to the wild-type stem cell transplant controls, one third ($n = 2$ of 6) of the gene-modified stem cell transplants using the UCOE promoter failed to correct the phenotype, with a similar failure frequency for the spleen focus forming (SFFV) promoter. Our experiments and those of Pike-Overzet et al have been performed in the context of the same national and European-funded projects in the same mouse strain in the same period of time in the very same animal facility. Therefore, we exclude that environmental differences in animal health and microbial state explain the seemingly divergent results. The key differences in experimental approach are reflected by (1) a

thorough analysis of phenotype correction failures in our study and (2) up to 18-month observation of the mice in contrast to at most 4.6 months reported by Pike-Overzet et al.

Pike-Overzet et al should have performed a careful analysis of the phenotype correction failures. In the discussion, it is stated that “a number of mice did not survive, most likely due to bone marrow failure”; however, there are no data on bone marrow chimerism required to substantiate such an explanation. Bone marrow failure should not occur in syngeneic mouse stem cell transplants, unless, of course, the gene transfer procedure resulted in large losses of repopulating stem cells. Unfortunately, stem cell recovery after transduction was not addressed either.

The development of efficacious and safe gene therapy protocols for *RAG1* severe combined immunodeficiency (SCID) requires long-term follow-up studies of relatively large cohorts of gene therapy-treated mice, transplantation of secondary recipients to monitor sustained correction of the phenotype and potential adverse effects, and a detailed vector integration analysis and should include autoimmune parameters, such as T-cell activation markers, generation and suppressive function of regulatory T cells, IgE levels, detection of autoimmune antibodies, and T-cell infiltrations in skin and liver, supplemented with careful analysis of epithelial thymic reconstitution in parallel with evaluation of germinal centers in treated spleen.³ Moreover, evaluation of B-cell distribution and function is crucial to define the restored immune competence after gene therapy.^{4,5}

We immediately agree that optimal efficacy requires a threshold expression level of the therapeutic transgene, below which efficacy rapidly goes down, as we previously documented for all murine models for other monogenic diseases investigated, which include *Rag2*^{-/-}, X-SCID, and Pompe mice. Therefore, part of the safety evaluation of any stem cell gene therapeutic approach should be an accurate estimate of the minimum VCN and cell dose required for optimal efficacy. Also, this has not been addressed at this stage in *Rag1*^{-/-} mice.

Genetic background and environmental factors play an important role in the clinical presentation of patients with hypomorphic mutations of SCID⁶ as well as *Rag2*^{R229Q/R229Q} Omenn mice⁴ on a chimeric or syngeneic background, both presenting with a broad diversity in clinical phenotype, large differences in the level of cellular infiltration grade, IgE levels, and so forth, most mice not developing skin erythrodermia. However, all mutant mice show activated T cells even in the absence of any other obvious sign of autoimmunity.

Pike-Overzet et al¹ make reference to the well-organized *RAG1* gammaretroviral vector study by Lagresle-Peyrou et al, in which autoimmune reactions have not been noted, which may be attributable to environmental conditions and microbial state, genetic background, end-point parameters used, or the observation time limited to 6 months in both primary and secondary recipients, while emphasizing the high VCN needed and revealing the risk of genotoxicity inherent to the vector choice.

The occurrence of several cases of leukemia in the pioneering X-linked SCID gammaretroviral vector gene therapy trials has conclusively demonstrated the risk of clinical implementation based on the evaluation of a small number of mice monitored for a short time after transplantation.⁷ Although *RAG1* deficiency still has a long road to go in preclinical efficacy and safety evaluation of the potentially curative lentiviral gene therapy approach before