GENE THERAPY

Is *IL2RG* oncogenic in T-cell development?

Arising from: N.-B. Woods, V. Bottero, M. Schmidt, C. von Kalle & I. M. Verma *Nature* 440, 1123 (2006)

The gene *IL2RG* encodes the γ-chain of the interleukin-2 receptor and is mutated in patients with X-linked severe combined immune deficiency (X-SCID). Woods *et al.*[1] report the development of thymus tumours in a mouse model of X-SCID after correction by lentiviral overexpression of *IL2RG* and claim that these were caused by *IL2RG* itself. Here we find that retroviral overexpression of *IL2RG* in human $CD34^+$ cells has no effect on T-cell development, whereas overexpression of the T-cell acute lymphoblastic leukaemia (T-ALL) oncogene *LMO2* leads to severe abnormalities. Retroviral expression of *IL2RG* may therefore not be directly oncogenic — rather, the restoration of normal signalling by the interleukin-7 receptor to X-SCID precursor cells allows progression of T-cell development to stages that are permissive for the pro-leukaemic effects of ectopic *LMO2*.

The occurrence of leukaemia in three patients in a gene-therapy trial for X-SCID (refs 2–4) has highlighted an adverse effect of insertional mutagenesis as a delivery technique for the therapeutic gene. In our investigation, we expressed both *LMO2* and *IL2RG* transgenes in the same retroviral vector that was used in the clinical gene-therapy trials; those trials found that retroviral integration of the corrective *IL2RG* occurred near the locus of the *LMO2* oncogene[5]. We concluded that this integration may have upregulated the expression of *LMO2* and triggered the leukaemia cases in the gene-therapy trials.

Woods *et al.* used a murine model in which stem cells from wild-type or mutant *Il2rg*$^{-/-}$ mice were transduced with very high amounts of human *IL2RG* by means of a lentiviral vector[1]. They found that these transplanted mice developed a high incidence of tumours. To the best of our knowledge, however, *IL2RG* has never been reported to act as an oncogene in human T-ALL, and murine studies should be interpreted with caution when extrapolating to humans. Although T-cell development is very similar in mice and humans at the genome-wide level[6,7], there are differences in individual genes: for instance, mutant *Il2rg*$^{-/-}$ mice lack T, B and natural killer (NK) cells, whereas human X-SCID patients have normal numbers of B cells.

Mouse–human hybrid fetal thymus organ culture (FTOC) is often used to study human T-cell development *ex vivo*. In FTOC experiments, we found that *LMO2*-transduced cells expressed the early thymocyte marker CD1a less frequently than untransduced cells derived from the same culture (22% compared with 57%), whereas a higher proportion of *LMO2*-transduced cells retained the progenitor marker CD34 (12% compared with 2–4%, representative of five different FTOC experiments). Later, during T-cell development, this block was even more pronounced (58% of cells in the subsequent immature CD4 single-positive stage, compared with 22% in controls). These results indicate that progenitor cells ectopically expressing *LMO2* are severely hampered in T-cell development.

The percentage of immature CD4 single-positive (37% compared with 38%) and the later $CD4^+CD8^+$ double-positive cells (6% compared with 9%) was similar in untransduced and *IL2RG*-transduced populations, indicating that retroviral-mediated expression of *IL2RG* does not affect T-cell development. *LMO2*-transduced cells developed normally into B, NK and myeloid cells.

The very high expression of lentiviral transgenic *IL2RG* in the transplanted mice studied by Woods *et al.*[1] may have contributed to development of T-cell lymphomas. In contrast, IL2RG concentrations were slightly lower than normal in the human X-SCID trials after treatment using retroviral vectors. The phenotype of the mouse tumours is very immature and different from that of the T-ALL that occurred in the three X-SCID patients. Unfortunately, Woods *et al.* do not indicate whether the tumours were clonal, whether they expressed IL2RG, or whether JAK3 was activated; it is possible that the *IL2RG* gene might itself be mutated — for example, as a result of errors in lentiviral reverse transcription, but details of the insertion sites recovered are not given.

In our view, the retroviral-mediated expression of *IL2RG* in haematopoietic precursors does not represent a leukaemogenic event. This γ-chain is highly expressed in normal haematopoietic $CD34^+$ precursors and during all stages of T-cell development, whereas *LMO2* is only expressed in progenitor cells and the earliest thymocytes[6]. Furthermore, *IL2RG* expression in treated X-SCID patients is within the normal range[5]. Persistent activation of JAK3 was not detected in these patients, indicating that IL2RG function was normal in the setting of retroviral expression[5].

Taken together, these observations argue against *IL2RG* acting as an oncogene in human gene therapy, although our experiments cannot rule out the possibility that a therapeutic transgene might provide an inappropriate growth stimulus when expressed at high dose or at an inappropriate time. Instead, it is likely that restored *IL2RG* expression allows T-cell development to progress to stages at which *LMO2* would normally be completely downregulated but might contribute to leukaemogenesis if ectopically expressed.

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1. Woods, N.-B., Bottero, V., Schmidt, M., von Kalle, C. & Verma, I. M. *Nature* **440**, 1123 (2006).
2. Hacein-Bey-Abina, S. *et al. N. Engl. J. Med.* **348**, 255–256 (2003).
3. Marshall, E. *Science* **299**, 320 (2003).
4. Check, E. *Nature* **433**, 561 (2005).
5. Hacein-Bey-Abina, S. *et al. Science* **302**, 415–419 (2003).
6. Dik, W. A. *et al. J. Exp. Med.* **201**, 1715–1723 (2005).
7. Weerkamp, F., Pike-Overzet, K. & Staal, F. J. T. *Trends Immunol.* **27**, 125–131 (2006).

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X-SCID transgene leukaemogenicity

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Gene therapy has been remarkably effective for the immunological reconstitution of patients with severe combined immune deficiency[1–3], but the occurrence of leukaemia in a few patients has stimulated debate about the safety of the procedure and the mechanisms of leukaemogenesis[4]. Woods *et al.*[5] forced high expression of the corrective therapeutic gene *IL2RG*, which encodes the γ-chain of the interleukin-2 receptor, in a mouse model of the disease and found that tumours appeared in a proportion of cases. Here we show that transgenic *IL2RG* does not necessarily have potent intrinsic oncogenic properties, and argue that

the interpretation of this observation with respect to human trials is overstated.

The occurrence of lymphoproliferative disease in 3 out of a total of 20 patients with X-linked severe combined immune deficiency (X-SCID), treated in two separate gene-therapy trials, was surprising as it was not predicted from prior preclinical studies in mice. However, insertional mutagenesis leading to activation of proto-oncogenic transcription factors, such as LMO2, has since been shown to be important tumour-promoting events[6].

The possibility that *IL2RG* might itself be a contributor to oncogenesis was proposed after coincident retroviral insertion sites were found at the *IL2RG* and *LMO2* loci in a mouse tumour[7]. Woods *et al.*[5] show that 33% (5 out of 15) of C57BL6 X-SCID mice develop T-cell lymphomas after reconstitution with either X-SCID or wild-type bone-marrow stem cells transduced with lentiviral vectors encoding murine *IL2RG*; however, X-SCID mice engrafted with wild-type bone-marrow cells treated by mock transduction, or with a potentially neutral vector encoding a reporter gene, did not develop lymphomas (up to 1.5 years' follow-up). As there were no common genomic targets in the five mice with lymphoma and because the controls did not develop disease, the authors conclude that the lymphomas were not caused by insertional mutagenesis and that the therapeutic transgene itself is intrinsically oncogenic.

We challenge this interpretation on several grounds. First, molecular data are not presented on insertion sites, although the transgene copy number is high (which, in the context of a potent, ubiquitously active promoter, will increase the risk of inadvertent mutagenesis[8]): there is insufficient information to judge whether there are significant differences in copy number and numbers of infused cells between the experimental groups, and therefore insertional mutagenesis cannot be excluded. Second, C57BL6 mice are intrinsically prone to haematological malignancy. Third, analysis of the tumours themselves was incomplete: it is unclear whether they were clonal or oligoclonal, why one mouse with a thymic lymphoma did not contain vector, and whether transgene insertion in tumour cells resulted in upregulation of recognized oncogenes (which may be located at very large distances from the insertion site).

In addition, the implications of the findings of Woods *et al.* are overstated with respect to human gene-therapy trials for X-SCID. Their transgene is overexpressed to artificially high levels (several-fold) in murine bone-marrow and spleen samples, whereas this is not the case in human studies (or in leukaemic clones derived from patients). There is no evidence in human studies for constitutive activation of JAK3-dependent signalling, indicating that IL2RG is functioning normally in this context (Woods *et al.*[5] do not investigate this crucial point.)

We have tested the efficacy and toxicity of IL2RG-encoding transgenes in murine systems. Of 68 mice (26 X-SCID and 42 wild-type mice) reconstituted by gammaretroviral or lentiviral gene transfer and monitored for more than 6 months, only 3 cases of lymphoma were identified (refs 9, 10, and previously unpublished results). Transgene sequences were found in the tumour mass in one of these, although the relation of tumour development to gene transfer was not investigated. Of the other two tumours, one was transgene-negative and the other was not evaluated (the tissue was inadvertently discarded before it could be analysed). Finally, of two newly generated transgenic lines in which human *IL2RG* is expressed from a *CD2* promoter in wild-type mice, 54 out of 54 mice have survived for more than 12 months without developing tumours.

Our results therefore undermine the conclusions of Woods *et al.*[5] and do not support the contention that *IL2RG* itself is potently oncogenic. Clinical gene-therapy trials are scrutinized by the regulatory authorities and by the public, so any data pertaining to these need to be rigorously underpinned before they can be properly informative.

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METHODS

Bone-marrow cells from X-SCID or wild-type mice (C57BL6) were transduced with replication-defective retroviral vectors encoding *IL2RG* complementary DNA and engrafted into irradiated (γ-chain-deficient; $n = 26$) or wild-type ($n = 42$) recipients. Recipient animals comprised the following overlapping experimental groups: murine *IL2RG* transgene ($n = 22$); human *IL2RG* transgene ($n = 46$); gammaretroviral vector with intact long terminal repeats ($n = 56$); lentiviral vector (internal spleen focus forming virus promoter; $n = 12$); wild-type bone marrow ($n = 42$); X-SCID bone marrow ($n = 26$). All mice were analysed periodically between 6 and 12 months after engraftment for immunophenotypic and histological abnormalities. Additionally, bone marrow from three successfully treated X-SCID animals (gammaretroviral vector, human *IL2RG*) was transplanted into a group of eight secondary recipients and followed for up to 6 months, during which time no tumours developed (not included in totals). For generation of transgenic lines, a human *IL2RG* construct (CD2 promoter) was microinjected into C57BL6 × CBA/Ca F2 fertilized eggs. Two founder animals with a confirmed integrated transgene were used to generate two independent lines. Further details of vectors and methodology are available from the authors.

1. Cavazzana-Calvo, M. *et al. Science* **288**, 669–672 (2000).
2. Aiuti, A. *et al. Science* **296**, 2410–2413 (2002).
3. Gaspar, H. B. *et al. Lancet* **364**, 2181–2187 (2004).
4. Hacein-Bey-Abina, S. *et al. N. Engl. J. Med.* **348**, 255–256 (2003).
5. Woods, N.-B., Bottero, V., Schmidt, M., von Kalle, C. & Verma, I. M. *Nature* **440**, 1123 (2006).
6. Hacein-Bey-Abina, S. *et al. Science* **302**, 415–419 (2003).
7. Dave, U. P., Jenkins, N. A. & Copeland, N. G. *Science* **303**, 333 (2004).
8. Modlich, U. *et al. Blood* **105**, 4235–4246 (2005).
9. Soudais, C. *et al. Blood* **95**, 3071–3077 (2000).
10. Otsu, M. *et al. Mol. Ther.* **1**, 145–153 (2000).

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Woods et al. *reply*

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Using a lentiviral vector and a murine model of X-linked severe combined immune deficiency (X-SCID), we have shown that mice transplanted with cells constitutively overexpressing the therapeutic gene *IL2RG*, a subunit found in several cytokine receptors, can develop thymic lymphomas 6 to 12 months later[1]. Our results contradict those of Pike-Overzet *et al.*[2] and Thrasher *et al.*[3].

We do not claim that insertional mutagenesis

plays no part in this oncogenesis. We have analysed integration sites of lymphomic *IL2RG*-transduced mice tissues[1] and found dominant clones in thymuses that have integration profiles similar to those seen in the spleen and/or bone marrow of the individual animals; this suggests that the lymphoma is clonal. However, we found no common integration sites among the mice, as was seen in the X-SCID patients affected in the gene-therapy trial, where three out of three patients had insertional gene activation of the *LMO2* gene locus. Also, as none of the mice originally transplanted with a lentiviral control vector developed lymphomas[1], we maintain that insertional mutagenesis was not the only oncogenic event. Moreover, a lentiviral vector construct similar to the one we used has recently been shown to be significantly less prone to causing insertional mutagenesis than the oncoretroviral vectors that drive expression from the long terminal repeats[4].

IL2RG transgene expression was high in our mice that developed lymphomas[1], with even the lowest amount of expression being several-fold higher than in normal transplanted bone marrow. Previously, lymphomas in X-SCID-treated mice were rarely found, probably because of the short duration of studies. And oncoretroviral expression of *IL2RG* was on average normal to subnormal[5–9], unlike in our transplanted mice (33% of which developed lymphomas)[1]. In secondary mice transplanted from three primary retrovirus transgene-treated donors, expression of *IL2RG* was below endogenous levels[5]. Although the discrepancy with our results[1] may be ascribed to our much longer follow-up period (up to 1.5 years), it is possible that lymphoma development may have been linked with the increased expression of *IL2RG* in our mice.

Our results should be interpreted with caution and not be extrapolated into drastically changing or terminating currently successful gene-therapy protocols. If increased expression of *IL2RG* is potentially oncogenic in mice, an additional safety measure would be to ensure that *IL2RG* transgene expression is physiological. The mouse model may not necessarily reflect the patient setting, however, as there are significant differences between mice and humans in signalling by some of the IL2RG-containing interleukin receptors. IL2RG signals through the activation of the JAK3 molecule and we are currently analysing samples of lymphoma tissue from our mice for the presence of activated JAK3, which would provide further evidence that *IL2RG* in this context is contributing to the development of the tumours. In two of the three patients who developed lymphoma after gene therapy, however, there was no constitutive activation of JAK3 in their tumours.

Clinical experience in treating X-SCID patients with retroviral vectors that express *IL2RG* is increasing. Although a significant number of *IL2RG*-expressing T-cell clones exist in each treated patient because each of their T cells is transgenic, more than 5 years of follow-up have been uneventful in several cases. Also, no *IL2RG*-related lymphoproliferation has been reported in humans in the absence of *LMO2* activation. However, oncoretroviral vector expression from the long terminal repeat is subject to positional variegation, and effects on transgene expression can occur that are dependent on the site of integration. For example, a therapeutic retrovirus vector might land in a chromosomal site where expression of the transgene is enhanced, leading to overexpression.

Our intention was to warn other investigators that the *IL2RG* gene, when overexpressed in some settings, has the potential to be oncogenic — a finding that is not altogether surprising for a transgene encompassing the signalling portion of a growth-factor receptor. Further details, including the clonality of these lymphomas and quantification of the expression of IL2RG, and analysis of downstream signalling pathways, will shortly be submitted for publication.

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1. Woods, N.-B., Bottero, V., Schmidt, M., von Kalle, C. & Verma, I. M. *Nature* **440**, 1123 (2006).
2. Pike-Overzet, K. *et al. Nature* **443**, doi:10.1038/nature05218 (2006).
3. Thrasher, A. J. *et al. Nature* **443**, doi:10.1038/nature05219 (2006).
4. Montini, E. *et al. Nature Biotechnol.* **24**, 687–696 (2006).
5. Soudais, C. *et al. Blood* **95**, 3071–3077 (2000).
6. Lo, M. *et al. Blood* **94**, 3027–3036 (1999).
7. Otsu, M. *et al. Mol. Ther.* **1**, 145–153 (2000).
8. Otsu, M., Sugamura, K. & Candotti, F. *Blood* **97**, 1618–1624 (2001).
9. Avilles Mendoza, G. J. *et al. Mol. Ther.* **3**, 565–573 (2001).

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