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To cite this version:

Oumeya Adjali, Gilles Marodon, Marcos Steinberg, Cédric Mongellaz, Véronique Thomas-Vaslin, et al.. In vivo correction of ZAP-70 immunodeficiency by intrathymic gene transfer.. Journal of Clinical Investigation, American Society for Clinical Investigation, 2005, 115 (8), pp.2287-95. <10.1172/JCI23966>. <hal-00113038>

HAL Id: hal-00113038
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Submitted on 10 Nov 2006

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In vivo correction of ZAP-70 immunodeficiency by intrathymic gene transfer

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SCID patients have been successfully treated by administration of ex vivo gene-corrected stem cells. However, despite its proven efficacy, such treatment carries specific risks and difficulties. We hypothesized that some of these drawbacks may be overcome by in situ gene correction of T lymphoid progenitors in the thymus. Indeed, in vivo intrathymic transfer of a gene that provides a selective advantage for transduced prothymocytes should result in the generation of functional T lymphocyte progeny, allowing long-term immune reconstitution. We assessed the feasibility of this approach in a murine model of ZAP-70–deficient SCID. A T cell–specific ZAP-70–expressing lentiviral vector was injected into thymi of adult ZAP-70−/− mice without prior conditioning. This resulted in the long-term differentiation of mature TCR-αβ thymocytes, indicating that the vector had integrated into progenitor cells. Moreover, peripheral ZAP-70–expressing T cells demonstrated a partially diversified receptor repertoire and were responsive to alloantigens in vitro and in vivo. Improved treatment efficacy was achieved in infant ZAP-70−/− mice, in which the thymus is proportionately larger and a higher percentage of prothymocytes are in cycle. Thus, intrathymic injection of a lentiviral vector could represent a simplified and potentially safer alternative to ex vivo gene-modified hematopoietic stem cell transplantation for gene therapy of T cell immunodeficiencies.

Introduction

SCID is a heterogeneous group of genetic disorders that is almost universally fatal in infancy, due to the advent of opportunistic infections. SCID can be treated by allogeneic stem cell transplantation, but the majority of patients do not have histocompatible donors. In the absence of histocompatible donors, SCID patients sometimes receive an HSC transplant from HLA-haploidentical donors. Although recent modifications of this protocol have increased survival to levels approaching 75%, there are significant short-term and long-term complications, and the emergence of significant numbers of circulating naïve T cells often requires more than 150 days (1–3). Alternatively, SCID represents a unique favorable setting for gene therapy strategies as the gene-corrected lymphocytes should have a selective advantage. Indeed, such an approach has already been shown to be beneficial in several adenosine deaminase–deficient (ADA-deficient) and γc-deficient SCID patients (4–7). The extensive ability of T progenitors to undergo massive expansion translates into the possibility that only a few corrected progenitor cells can reconstitute the T cell compartment, as highlighted by patients in whom a spontaneous reversion mutation resulted in a “natural” gene therapy. Specifically, several individuals with inherited mutations that are known to result in SCID (X-SCID and ADA-deficiency) have been found to be relatively healthy, with relatively normal T cell numbers (8–11). In each of these patients, this was likely due to a reversion mutation, and because this event is statistically improbable, it likely occurred in a single hematopoietic progenitor/stem cell (8–10). These natural gene therapy experiments strongly suggest that gene correction of a single progenitor cell that is capable of proliferation, differentiation, and migration can potentially eliminate the critical symptoms associated with SCID.

Based on the findings that correction of a single or few progenitor cells can restore immune reconstitution, clinical gene therapy trials for SCID disorders have thus far relied on ex vivo gene transfer into HSC/progenitor cells (CD34+) using nonspecific retroviral vectors. Despite its proven efficacy (4, 6, 7, 12, 13), such an approach carries specific risks and difficulties (14). First, as gene transfer with murine leukemia virus–based retroviral vectors cannot occur in the absence of mitosis, this approach requires an ex vivo culture of CD34+ cells in the presence of a cocktail of cytokines. This is a cumbersome manipulation that can affect the long-term function of these cells. Second, the ectopic expression of the therapeutic gene in all stem cell progeny could generate serious side effects, notably when the transgene participates in signal transduction pathways. Finally, oncoretroviral insertion per se, by modifying the expression of cellular genes, can contribute to leukemogenesis (14, 15).

We hypothesized that some of these drawbacks could be overcome by in situ gene correction of T lymphoid progenitors in the thymus using T cell–specific lentiviral vectors. This hypothesis was supported by the following observations: (a) lentiviral vectors have resulted in efficient in vivo gene transfer in hepatocytes, antigen-presenting cells, and muscle cells as well as cells in the

Nonstandard abbreviations used: ADA, adenosine deaminase; BV, β chain hyper-variable region; CD34, complementarity determining region 3; DN, double negative; DP, double positive; eGFP, enhanced GFP; FSC, forward angle scatter; IRES, internal ribosome entry site; IT, intrathymic(ally); pT-ZAP, T cell–specific lentiviral vector encoding human ZAP-70; SP, single positive; SSC, side angle scatter; TCRBV, TCR β chain hyper-variable region; TU, transduction unit.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J. Clin. Invest. 115:2287–2295 (2005). doi:10.1172/JCI23966.
central nervous system (16–21); (b) intrathymic (IT) injection of a nonspecific lentiviral vector results in the transduction of thymic epithelial cells as well as a low number of immature thymocytes (22); (c) early T lymphoid progenitors (ETP) in the thymus appear to sustain production of T lineage progeny for longer periods of time than do the common lymphoid progenitors (CLPs) found in the BM (23). Thus we reasoned that IT injection of a T cell–specific lentiviral vector encoding a gene that provides a selective advantage for transduced prothymocytes might result in the generation of functional T lymphocyte progeny with subsequent long-term immune reconstitution.

The feasibility of this approach was assessed in a murine model of ZAP-70 deficiency. ZAP-70 is a 70-kDa protein tyrosine kinase (PTK) that is recruited to the TCR following antigenic stimulation of the TCR (24). It is expressed at approximately equivalent levels in thymocytes, mature T cells, and NK cells (25). Its absence results in a SCID phenotype with a block in T cell development at the CD4+CD8+ thymocyte stage (26–28). To obviate the potential obstacles/risks concerning ubiquitous expression of ZAP-70, the human WT gene was introduced into a T cell–specific lentiviral vector (29). Here we demonstrate that direct IT injection of this ZAP-70–expressing lentiviral vector results in the reconstitution of polyclonal and functional T cells.

Results

Thymocyte development in mice injected IT with a ZAP-70–expressing lentiviral vector. In an attempt to reconstitute T lineage cells via in situ gene transfer, we injected a T cell–specific lentiviral vector encoding human ZAP-70 (pT-ZAP) directly into the thymi of 8- to 12-week-old ZAP-70–deficient mice. In pT-ZAP, the ZAP-70/internal ribosome entry site/enhanced GFP (ZAP-70/IRES/eGFP) cassette is under advantage for transduced prothymocytes might result in the generation of functional T lymphocyte progeny with subsequent long-term immune reconstitution.

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The absence of ZAP-70 is associated with a relatively late block in T cell differentiation at the CD4/CD8 double positive (DP) thymocyte stage (26–28) (Figure 2A). As expected from the very low MOI used, the actual percentage of transduced thymocytes was extremely low (0.3%; Figure 2B), and thus IT injections of pT-ZAP were not expected to dramatically alter the overall percentage of single positive (SP) thymocytes as compared with the overall percentage of SP cells in deficient animals (Figure 2A). However, while TCR-β upregulation is blocked in ZAP-70–deficient mice, IT-injected mice demonstrated a significant increase in TCR-β+ CD4 SP thymocytes (data not shown). Moreover, analysis of eGFP–TCR-β+ cells showed that percentages of mature CD4 and CD8 SP thymocytes were similar to those detected in WT mice, representing more than 80% of cells (Figure 2B). In marked contrast, the phenotype of thymocytes within the eGFP–TCR-β+ population of pT-ZAP–injected mice was largely that of immature DP cells (71%, Figure 2B). These results indicate that ZAP-70 expression driven from pT-ZAP is capable of correcting the differentiation block in ZAP-70 deficiency.

The activation status of these T lymphocytes was determined by assessing the expression of the CD25 and CD69 activation markers. T cells from ZAP-70–/– mice injected IT with pT-ZAP expressed the CD25 activation marker on a significantly higher proportion of cells compared with the overall percentage of cells detected in WT mice (22% ± 4.6% [n = 5], P = 0.001; Figure 4B). Expression of the CD69 activation marker was also elevated in ZAP-70–transduced T cells as compared with T cells in WT mice, with the elevated expression being indicative of T cell activation. While both naive and memory phenotype (32) T cell populations were observed in reconstituted mice, the percentage of naive T cells (CD62L+) was lower in mice injected IT with pT-ZAP (Figure 4B). Indeed, the overall percentage of peripheral T cells detected in pT-ZAP–injected mice was always lower than that observed in WT animals (Table 1). CD3+ lymph node T cells in the pT-ZAP–injected mice reached 26% whereas those in WT mice averaged approximately 60% (Table 1). Of note, the level of eGFP expression in the CD3+ T cells varied widely, from 14 to 66%. As T cell levels in ZAP-70–/– mice generally do not exceed 4%, it is extremely
likely that the vast majority of T cells detected in the pT-ZAP–injected mice differentiated due to the presence of the ZAP-70 transgene. Thus, our inability to detect eGFP in all T cells is either due to some discordance between ZAP-70 and eGFP expression in vivo in the context of the upstream IRES (Figure 1 and Table 1) or to low downregulated levels of eGFP not detected by FACS.

ZAP-70 expression and diversity of T cells that developed in mice injected IT with a ZAP-70–expressing lentiviral vector. We next assessed the level of ZAP-70 expressed in peripheral T lymphocytes as well as the diversity of their TCR repertoire (Figure 5). Levels of lentiviral-encoded ZAP-70 were similar to those of the endogenous protein in WT mice. The ectopic expression of the ZAP-70 transgene was further studied by sorting CD3⁺ cells and CD3⁻ cells from WT mice and ZAP-70⁻/⁻ mice reconstituted by IT injection of pT-ZAP. Importantly, ectopic ZAP-70 expression was detected almost exclusively in the CD3⁺ T cell compartment upon expression from the pT-ZAP lentiviral vector (Figure 5A). Thus, the CD4 promoter appears to drive expression of ZAP-70 in T cells to levels similar to that of the endogenous promoter.

We then investigated the relative usage of each TCR β chain hypervariable region (TCRBV) within the global T cell population by flow cytometry and the Immunoscope/spectratype method (33, 34). Using this combination of methods, we established both the frequencies of β chain hypervariable region–expressing (BV-expressing) cells and their repertoire diversity. The Immunoscope method is based on an RT-PCR of the hypervariable region (TCRBV) within the global T cell population. The representative IT-reconstituted mouse presented here was sacrificed at 8 weeks after injection.

The CDR3 length profiles of T cells from the ZAP-70⁻/⁻ mice reconstituted by IT injection had a Gaussian distribution for an average of 26% of the 14 BV families analyzed (with a range of 0 to 75%), with the remaining BV families appearing as monoclonal or oligo-clonal. This is in contrast with WT mice, in which a Gaussian distribution was found for all the TCRBV families analyzed (Figure 5B). Flow cytometry analyses of TCRBV expression in 6 reconstituted mice also showed a diverse, though not normal, repertoire in both CD4 and CD8 T cell populations, with BV representation varying for each ZAP-70⁻/⁻ mouse reconstituted by IT injection. We found that, on average, 60% of the 11 BV families analyzed were detected by flow cytometry using BV-specific antibodies (with a range of 30% to 100%) and 77% by the Immunoscope method (with a range of 50% to 100%). This difference is likely due to the higher sensitivity of the Immunoscope method as compared with flow cytometry. Our results thus show that the T cell repertoire of pT-ZAP–injected mice was fairly diverse in terms of BV usage but was perturbed with respect to both frequencies and clonality.

Function of T cells that developed in mice injected IT with a ZAP-70–expressing lentiviral vector. The functionality of the T lymphocytes that developed in IT-injected ZAP-70⁻/⁻ mice was assessed in several ways, including by their ability to respond to ex vivo TCR stimulation (Figure 6A). Notably, ZAP-70 plays a critical role in the activation of mature T lymphocytes. In the absence of the WT protein, early as well as late biological responses such as proliferation are defective (35). In the absence of stimulation, neither WT nor pT-ZAP–transduced T cells significantly proliferated, showing that ectopic expression of ZAP-70 did not modulate the basal proliferative status of corrected T cells. Following 3 days of stimulation with either concanavalin A or α-CD3/IL-2, a significant percentage of both WT and pT-ZAP–transduced T cells had undergone up to 4 divisions. In contrast,
no division was induced in splenocytes isolated from control ZAP-70–deficient mice. Thus, pT-ZAP–transduced T cells, like WT T cells, are capable of responding to a TCR-specific stimulus.

To assess whether these pT-ZAP–transduced T cells were capable of responding to immune stimulation in vivo, mice were grafted with both syngeneic (C57BL/6) and fully mismatched allogeneic (BALB/c) skin grafts on the 2 sides of the back. Control mice included untreated ZAP-70–deficient mice (n = 3) and WT C57BL/6 mice (n = 2) transplanted with the same skin grafts. As expected, the untreated ZAP-70–deficient mice, who do not develop T cells, rejected neither the syngeneic nor the allogeneic grafts. In the control C57BL/6 mice, allograft rejection was acute, with signs of necrosis appearing from day 12 onward while syngeneic grafts were maintained. In the pT-ZAP–injected mice, signs of rejection were observed in 2 of the 3 mice between days 21 and 30. Importantly, the 2 mice who presented with erythema and thickening of the allogeneic graft had higher percentages of CD3+ peripheral T cells (16% and 19% splenic T cells, respectively) than the mouse without evidence of rejection (7%). So the grafts could be more precisely analyzed, they were biopsied between days 30–40, and histological analyses were performed on paraffin sections. In the syngeneic and allogeneic sections transplanted on the pT-ZAP–injected mice, there was evidence of fibroblast hyperplasia. However, only in allogeneic sections was lymphocyte infiltration prevalent in the superficial dermis and epidermal layers (in the 2 of 3 mice with clinical evidence of rejection; Figure 6B). Moreover, the T lymphocytes from the IT-injected mice proliferated in response to these alloantigens, as observed in a mixed lymphocyte reaction. Lymph node T cells from WT (C57BL/6) and skin-grafted pT-ZAP–reconstituted mice proliferated in response to allogeneic BALB/c antigens (Figure 6C).

<table>
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<th>Mice</th>
<th>Age at injection (weeks)</th>
<th>Time after injection (weeks)</th>
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<th>eGFP+ T cells (%)</th>
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<td>/</td>
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<td>2</td>
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<td>/</td>
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Discussion

Altogether, our data demonstrate that direct injection of T cell–specific lentiviral vectors into thymi of ZAP-70–deficient mice allows long-term T cell reconstitution and transgene expression in peripheral T lymphocytes. The efficacy of this treatment is in large part due to the context of SCID where the transgene confers a strong selective advantage (4–7). Importantly, this selective advantage is fully exploited by IT injection; we failed to observe T cell reconstitution upon i.v. injection of the pT-ZAP vector (O. Adjabi, M. Steinberg, and N. Taylor, unpublished observations).

Transduction of early T cell progenitors by IT lentiviral vector injections. Upon IT gene transfer, it was possible that the peripheral T lymphocytes in pT-ZAP–treated mice arose from the expansion of a clonal or oligodendral population of mature thymocytes derived from DP thymocytes.
transduced after β chain rearrangement. Our results argue against this hypothesis. Indeed, we previously reported that transduced DN cells, which contain the immature T cell progenitors, can be detected shortly after IT lentiviral injections of a nonspecific (21) or a T cell–specific lentiviral vector (our unpublished observations). Moreover, gene-modified thymocytes were detected for significant time periods, albeit at small numbers and in a low percentage of animals (<25%). In 1 pT-ZAP–injected animal who was maintained for 1 year, immature DN and DP thymocytes expressing eGFP (0.5%) were still detected at 1 year (Supplemental Figure 4). Nevertheless, it is not clear whether this is due to the maintenance of progenitor cells in the thymus or, more likely, to the recirculation of transduced progenitors into the thymus. The second hypothesis is supported by our finding that transduced lineage-negative progenitor cells can be detected in the bone marrow at more than 8 weeks following injection of pT-ZAP into the thymus (our unpublished observations). Thus, it will be important to assess the fate of thymic progenitors and determine whether a subpopulation of these cells can emigrate from the thymus and then reenter at later time points.

T cell diversity and function. The Immunoscope method is based on an RT-PCR of the hypervariable CDR3 and allows the lengths of the mRNA encoding each β chain of the TCR to be analyzed. In a normal setting, each peak is composed of multiple differently rearranged sequences. The detection of single peaks for some BV products was generated by reverse transcription with different TCRBV subfamily-specific primers and 1 Cβ primer; this was followed by a run-off reaction with a fluorescent Cβ primer. The histograms represent fluorescence intensity in arbitrary units (y axis) plotted against CDR3 size (x axis). Representative results showing the size distributions within 14 TCRBV families are shown.

Profiles detected in the IT-treated ZAP−/− animals is reminiscent of that of an X-SCID patient whose T cells developed from a reverse mutation in a single T cell precursor (9), resulting in a fairly positive clinical condition (37). Thus, the rescue of few progenitor/stem cells can promote the differentiation of T cells with a partially diversified TCR repertoire, sufficient to provide individuals with protection from infection and a positive clinical outcome.

Potential safety advantages of in situ gene therapy for SCID. It is noteworthy that in mice injected with the vector utilized here wherein eGFP was driven from a minimal CD4 promoter, eGFP expression was not detected in thymic stroma or liver, which were the main targets transduced following IT injection using ubiquitous lentiviral vectors (22). In general, this is a major advantage of lentiviral vectors, wherein lineage specificity can be obtained and contributes to the overall safety of the approach. Another advantage of our approach is that gene transfer was obtained in the absence of prior conditioning. Indeed, the vast majority of studies transplanting gene-modified HSC into murine SCID models were performed in lethally irradiated mice (31, 38, 39). Moreover, in human patients with ADA deficiency, a high level of immune reconstitution following injection of ex vivo gene-correlated HSC has been shown to require pretransplantation chemotherapy (6). Importantly, in ZAP−/−deficient mice, T cell reconstitution by HSC corrected ex vivo with a ZAP-70 retroviral vector does not occur in the absence of conditioning (O. Adjali, M. Steinberg, and N. Taylor, unpublished observations). Safety of the IT gene transfer protocol in terms of potential insertional mutagenesis or autoimmunity remains to be fully investigated but is likely to be diminished by the low MOI used in the in situ injections. Altogether though, we believe that the IT injection of T cell–specific lentiviral vectors without preconditioning represents a safety improvement for SCID gene therapy.

Perspectives. We show that a higher frequency of reconstitution was obtained in infant as compared with adult mice, further highlighting the potential of our approach in ZAP−/−-deficient children. In view of a potential clinical application, we recently assessed this approach in macaques, performing direct IT injection under ultrasound guidance (P. Moullet [INSERM, Nantes, France], personal communication) and determined it to be a simple and feasible technical gesture. We believe that the overall efficacy of our approach will be significantly improved in a clinical setting. Indeed, IT gene transfer of lentiviral vectors is not optimal when applied to mice for the following reasons: (a) at similar MOIs and at equivalent levels of activation, murine T cells are less susceptible to lentiviral infection than are human T cells (our unpublished observations and ref. 36); and (b) the small size of the mouse thymus allows neither the inoc-
tion of large quantities of vectors nor repeat injections. Likewise, while the translation of gene therapy from mice to larger animals or even humans has generally resulted in decreased efficiencies, we believe that this would not be the case in this setting. Thus, gene transfer using T cell–specific lentiviral vector calls for further development of this strategy for the treatment of SCID.

**Methods**

*Lentiviral construction and virus production.* The plasmid pGM1 encoding for a T cell–specific lentiviral vector expressing eGFP has been previously described (29). To obtain a T cell–specific lentiviral vector encoding ZAP-70 and eGFP, a blunted–BamHI/SalI ZAP-70/IRES/eGFP insert from plasmid LZRS pBMN ZAP-70/IRES/eGFP (40) was inserted between EcoRV-SalI of the pGM1 plasmid (29) in replacement of the original eGFP coding sequences. The ensuing T-cell–specific lentiviral vector pRRLSIN/cPPT/CD4pmE/ZAP-70/IRES/eGFP/WPRE is hereafter referred to as pT-ZAP.

Virions were produced by transient calcium phosphate cotransfection of 293T cells with the vector plasmid, an encapsidation plasmid lacking Vif, Vpr, Vpu, and Nef accessory HIV-1 proteins (p8.91), and a vesicular stomatitis virus–G protein (VSV-G) envelope expression plasmid (pHCMV-G) (41). Viral titers, expressed as TUs, were determined by assessing transductions of Jurkat T cells with serial dilutions of virion preparations. Expression of ZAP-70 and eGFP from this cassette is proportional as assessed in vitro using the Jurkat T cell line (data not shown).

*Mice and IT injections. ZAP-70−/− mice,* kindly provided by A. Singer and R. Bosselut (NIH), were bred and maintained under pathogen-free conditions. IT injections were performed at either 2–3 weeks of age (infant mice) or 8–12 weeks of age (adult mice). In the former case, virions in a total volume of 10–30 µl were injected directly through the skin into the thoracic cavity immediately above the sternum, using a 0.3-ml, 28-gauge, 8-mm insulin syringe. Adult mice were anesthetized with 40 mg/kg of

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**Figure 6**

In vitro and in vivo responsiveness of T lymphocytes reconstituted by IT injection of pT-ZAP. **(A)** Splenocytes from ZAP-70−/−, WT, and IT-reconstituted ZAP-70−/− mice were labeled with the fluorescent dye CFSE and cultured in vitro in the presence of concanavalin A (Con A) or αCD3/IL-2 for 3 days. Cells were analyzed for CFSE intensity by flow cytometry. The numbers shown above the peaks indicate the number of cell divisions. The IT-reconstituted ZAP-70 mice were sacrificed at 8 weeks after injection. **(B)** Histological sections of syngeneic (C57BL/6) and allogeneic (BALB/c) skin sections, which had both been grafted on the same IT-reconstituted ZAP-70 mouse, were stained with H&E. The presence of lymphocytes (shown as dark purple staining) infiltrating into the epidermis is shown in the enlarged inset. The IT-reconstituted mouse was injected 14 weeks prior to skin grafting, and skin histology was performed 30 days later. Magnification, ×200. **(C)** Lymph node cells from WT (C57BL/6) and IT-reconstituted ZAP-70 mice (pregrafted as described above; sacrificed at 8 weeks after graft) were labeled with CFSE and cultured in the absence or presence of allogeneic BALB/c splenocytes. After 3 days, cells were stained with an αCD4 mAb, and the percentages of CD4+ cells that divided, as assessed by a loss of CFSE intensity, are indicated in each dot plot. MLR, mixed lymphocyte reaction.
pentobarbital. Midincision of the lower neck was performed to gain access to the trachea. Incision of the sternum was performed on the first 2 ribs, and virions were injected into the visualized thymus. All experiments were approved by the Institutional Review Board at the Animal Facility of the Institut de Génétique Moléculaire de Montpellier.

**Immunophenotyping, proliferation, and flow cytometry analyses.** ZAP-70−/− mice were killed and dissected 6–18 weeks after injection. Controls were obtained from age-matched 129/Sv mice. Cells from lymph nodes, spleen, and thymus were stained with the indicated conjugated antibodies (BD Biosciences — Pharmingen). For proliferation analyses, splenocytes were resuspended at a concentration of 2.5 × 10^6 cells/ml and labeled with the fluorochrome conjugated mAb. The CD4/CD8 distribution within the fractions of Ki67+ thymocytes is shown. (C) T cell reconstitution in the lymph node of a ZAP-70 KO infant mouse injected IT with pT-ZAP was monitored by flow cytometry and is representative of 15 of 20 IT-treated infant mice.

**Figure 7**
Enhanced T cell reconstitution efficacy following IT injection of a pT-ZAP in infant mice. (A) The FSC and SSC profiles of thymi from a 2-week-old (infant) and adult ZAP-70 KO mouse are shown. (B) Cell cycle entry of total thymocytes from an infant and adult ZAP-70 KO mouse was monitored by assessing intracellular expression of the Ki67 antigen using a fluorochrome-conjugated mAb. (C) The CD4/CD8 distribution within the fractions of Ki67+ thymocytes is shown. (D) Cell cycle entry of total thymocytes from an infant and adult ZAP-70 KO mouse was monitored by flow cytometry and is representative of 15 of 20 IT-treated infant mice.

**Statistical analyses.** Statistical significance was determined using a 1-tailed paired Student’s t test. P values of less than 0.05 were considered to be statistically significant. All data are presented as means ± SD.

**Acknowledgments**
We thank N. Noraz, S. Choquart, R. Vicente, and V. Zimmermann for their critical input and assistance during the course of this study (45).

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**Pro version 4.0.2 (BD) or FlowJo software version 4.6.1 (Tree Star Inc.).**

**Skin transplantation.** Skin grafts were performed on anesthetized WT (C57BL/6) and ZAP-70−/− mice as well as on ZAP-70−/− mice with reconstituted T cells 3 months after IT injection of pT-ZAP. Full-thickness skin grafts (0.5 cm^2) were prepared from the base of both syngeneic C57BL/6 and allogeneic BALB/c donor tails. Graft beds were prepared on the right and left lateral backs of recipient mice. Skin grafts from both C57BL/6 and BALB/c mice were attached to the backs of each mouse with interrupted sutures of silk thread, and graft appearance was monitored 3 times a week. Paraffin sections were prepared following formalin fixation of biopsied grafts. Sections were stained with H&E.

**TCR CDR3 size analyses.** Total RNA was prepared using RNAsure (Eurobio). Five µg of RNA was reverse transcribed using oligonucleotide (dT) primers (Pharmacia Biotech) and Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Corp.). cDNAs were amplified (cycles: 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C) using 2.5 IU of the AmpliTaq polymerase (Roche Diagnostics Corp.). A 50-µl reaction mixture with 1 of 14 TCRBV subfamily-specific primers and a Cβ primer that recognizes the constant regions Cβ1 and Cβ2 of the Cβ chain of the TCR. Primers are described in refs. 43 and 44, except for VB3 (GCAAGATGAGTTGTATCCCTG) and Cβ4 (GGCCATGGAACTGCACCTGG) primers (Proligo France SAS). The Cβ fluorescent probe was mCB6-ROX 5′-CTTGGGTGAGTCTACCTTCC-3′. The final concentration was 0.5 µM for each primer, 0.2-mM dNTPs, and 2-mM MgCl2 in 1× PCR Buffer (Roche Diagnostics Corp.). For each PCR, 2 µl was further processed in a run-off reaction (10 cycles: 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C) using 0.2 µM of a ROX-labeled Cβ primer (Proligo France SAS) and 2.5 IU of AmpliTaq polymerase (Roche Diagnostics Corp.). Each run-off product was denatured and loaded on a gel for fluorescence analysis using an Applied Biosystems ABI Prism 377 DNA Sequencer. To obtain the CDR3 length profiles depicted in Figure 6, raw data were analyzed using Immunoscope 3.01b software (LogInSiERM). The TCRBV nomenclature proposed by Arden et al. was used in this study (45).

**Acknowledgments**
We thank N. Noraz, S. Choquart, R. Vicente, and V. Zimmermann for their critical input and assistance during the course of this study (45).
this study. We are indebted to K. Chebbi for his scientific supervision of all our mouse experiments. We are grateful to G. Barneon and D. Antonelli for expert assistance with histological and TCR CDR3 size analyses, respectively. O. Adjali, G. Marodon, and M. Steinberg were supported by fellowships from the French Ministry of Higher Education, Association Française contre les Myopathies (AFM) and Fondation pour la Recherche Medicale, and AFM and Fundacion YPF, respectively. This work was supported by the Immune Deficiency Foundation, the AFM, the Association France-Israel pour la Recherche en Science et Technologie, the Association pour la Recherche sur le Cancer, March of Dimes grant 6-FY99-406, the Centre National de la Recherche Scientifique, and INSERM.

Received for publication November 22, 2004, and accepted in revised form May 31, 2005.

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