T-cell-receptor signalling in inositol 1,4,5-trisphosphate receptor (IP₃R) type-1-deficient mice: is IP₃R type 1 essential for T-cell-receptor signalling?

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Stimulation of T-cells via the T-cell receptor (TCR) complex is accompanied by an increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i). Recently, it was reported that a stable transformant of the human T-cell line, Jurkat, expressing an antisense cDNA construct of inositol 1,4,5-trisphosphate receptor (IP₃R) type 1 (IP₃R1), failed to demonstrate increased [Ca²⁺]_i or interleukin-2 production after TCR stimulation and was also resistant to apoptotic stimuli. This cell line lacked IP₃R1 expression, but expressed the type-2 and -3 receptors, IP₃R2 and IP₃R3 respectively [Jayaraman, Ondriasova, Ondrias, Harnick and Marks (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 6007–6011, and Jayaraman and Marks (1997) Mol. Cell. Biol. 17, 3005–3012]. The authors concluded that IP₃R1 is essential for TCR signalling and suggested that Ca²⁺ release via IP₃R1 is a critical mediator of apoptosis. To establish whether a loss of IP₃R1 function in T-cells occurred *in vivo* and *in vitro*, we investigated Ca²⁺ signalling

after TCR stimulation and the properties of T-cells using IP₃R1-deficient (IP₃R1^{−/−}) mice. As IP₃R1^{−/−} mice die at weaning, we transplanted bone marrow cells of IP₃R1^{−/−} mice into irradiated wild-type mice. Western blot analysis showed that the recipient IP₃R1-containing (IP₃R1^{+/+}) lymphocytes were replaced by the donor IP₃R1^{−/−} lymphocytes after transplantation and that expression of IP₃R2 and IP₃R3 was unaltered. In contrast with the previous reports, T-cells lacking IP₃R1 were able to mobilize Ca²⁺ from intracellular Ca²⁺ stores after stimulation via the TCR. We observed no significant differences between IP₃R1^{+/+} and IP₃R1^{−/−} T-cells in terms of the number of thymocytes and splenocytes, the proportion of the T-cell phenotype, proliferative response to anti-CD3 monoclonal antibody (mAb) stimulation and cell viability. Therefore IP₃R1 is not essential for T-cell development and function.

INTRODUCTION

Activation of T-cells by a specific antigen is a fundamental event that is essential for the immune system to protect the host against pathogens. Stimulation of the T-cell receptor (TCR) complex is accompanied by the following sequential steps: activation of protein tyrosine kinases (PTKs), recruitment of the SH2 (phosphotyrosine-binding) domain containing phospholipase C-γ (PLCγ) and activation of PLCγ by tyrosine phosphorylation, resulting in phospholipid hydrolysis yielding diacylglycerol and D-myo-inositol 1,4,5-trisphosphate (IP₃). Both of these latter molecules function as intracellular second messengers: diacylglycerol activates protein kinase C (PKC) [1], which exerts its physiological function through phosphorylation of target proteins, whereas IP₃ binds to IP₃ receptors (IP₃Rs) and induces Ca²⁺ release into the cytoplasm from intracellular Ca²⁺ stores [2]. Subsequently, the depletion of Ca²⁺ stores activates Ca²⁺ influx through plasma-membrane channels, which causes a sustained increase in the free intracellular [Ca²⁺]_i ([Ca²⁺]_i) and, in turn, leads to the activation of various transcription factors and the expression of T-cell-specific functions [3–5].

T-cells express three types of IP₃Rs: IP₃R type-1 (IP₃R1), -2 (IP₃R2) and -3 (IP₃R3) [6–8]. It is uncertain whether each type of IP₃R has a specific function in TCR signalling. It was reported recently that a stable transformant of the human T-cell line, Jurkat, expressing an antisense cDNA construct of IP₃R1 failed to increase [Ca²⁺]_i or produce interleukin-2 after TCR stimulation [9] and was also resistant to apoptotic stimuli [8]. Because this cell line lacked IP₃R1 but expressed IP₃R2 and IP₃R3, the authors concluded that IP₃R1 was essential for TCR signalling and suggested that intracellular Ca²⁺ release via IP₃R1 is a critical mediator of apoptosis.

Recently, we have generated IP₃R1-deficient (IP₃R1^{−/−}) mice using gene targeting. IP₃R1^{−/−} mice have a severely abnormal phenotype, which includes ataxia, epileptic seizures, small body size and death before weaning [10]. If IP₃R1 is essential for TCR signalling and apoptosis, we would expect IP₃R1^{−/−} mice to show abnormalities of T-cells and the immune system. For example, T-cells from these mice would lack both normal increases in [Ca²⁺]_i and cell proliferation after TCR stimulation and resistance to apoptosis should result in the failure of negative selection, thereby causing proportions of the

Abbreviations used: 7AAD, 7-aminoactinomycin D; BCR, B-cell antigen receptor; BM, bone marrow; [Ca²⁺]_i, free intracellular [Ca²⁺]; FITC, fluorescein isothiocyanate; IP₃, D-myo-inositol 1,4,5-trisphosphate; IP₃R(*n*), IP₃ receptor (type *n*; *n* = 1–3); mAb, monoclonal antibody; PE, phycoerythrin; TCR, T-cell receptor.

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T-cell phenotype in the thymus to be abnormal. To test this hypothesis and to establish the result of a loss of IP₃R1 function in T-cells, we have produced IP₃R1^{-/-} mice with a pure genetic background of C57BL/6 and then transplanted the IP₃R1^{-/-} bone marrow (BM) cells into irradiated C57BL/6 mice to study the properties of IP₃R1^{-/-} T-cells *in vivo* and *in vitro* in terms of T-cell phenotype, proliferative response, Ca²⁺ mobilization after TCR stimulation and cell viability.

MATERIALS AND METHODS

Reagents and antibodies

Monoclonal antibodies (mAbs) 18A10, KM1083 and KM1082 against IP₃R1, IP₃R2 and IP₃R3 respectively were prepared as described previously [6,11]. Anti-CD3 mAb 2C11 (Cedarlane Lab. Ltd., Hornby, ON, Canada), phycoerythrin (PE)-labelled anti-Thy1.2 mAb (53-2.1; Phar Mingen, San Diego, CA, U.S.A.) and PE-labelled anti-CD4 mAb (GK1.5, Phar Mingen) were purchased. Fluorescein isothiocyanate (FITC)-labelled anti-CD8 mAb (53.6.7) and FITC-labelled anti-B220 mAb (RA3/6B2) were obtained from American Type Culture Collection (Rockville, MD, U.S.A.).

IP₃R1^{-/-} mice and back-crossing

IP₃R1^{-/-} mice were generated using gene targeting [10]. In order to perform studies on the immune system, we produced (by back-crossing) IP₃R1^{-/-} mice with a pure C57BL/6 genetic background. Back-crossing was done using standard procedures [12]. The genetic background of the F2 generation of IP₃R1^{-/-} mice is composed of 129/Sv and C57BL/6 in the same ratio to each other. The 129/Sv background is derived from the J1 ES (embryonic stem) cell line, which was used to produce germ-line chimaeras in the process of generating IP₃R1^{-/-} mice. A male heterozygote F2 was mated with a female C57BL/6 mouse and its heterozygous male offspring were chosen for subsequent mating with female C57BL/6 mice; this process was repeated more than six times. On the first occasion that we obtained heterozygous female offspring during these repeats, another step was added in which the heterozygous female was mated with a C57BL/6 male to replace the Y-chromosome of F2 mice. After these crosses, in which the resultant background of a heterozygote was expected to be C57BL/6 in >99% of cases, we then conducted double heterozygote crossing and obtained homozygotes (IP₃R1^{-/-}) with a pure genetic background.

BM transplantation

Recipient 7-week-old male C57BL/6 mice received a lethal whole-body dose of radiation (1000 rad from a ¹³⁷Cs source) before the intravenous injection of 1×10^6 fresh, unseparated BM cells from either IP₃R1^{+/+} or IP₃R1^{-/-} male mice. All experiments using lymphocytes from the recipients were performed 7 to 11 weeks after transplantation.

Western blotting

Freshly isolated thymus and spleen were dispersed in RPMI 1640 medium [10% (v/v) fetal-calf serum] with a slide glass and passed through a 200- μ m mesh nylon screen to obtain thymocytes and splenocytes respectively. Thymocytes (1×10^7 cells) and splenocytes (1×10^7 cells) were solubilized in 100 μ l of lysis buffer [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA/1 mM 2-mercaptoethanol/10% (v/v) glycerol/1% (v/v) Triton X-100/0.1 mM PMSF/10 μ M leupeptin/10 μ M pepstatin

A] on ice for 30 min. The insoluble fraction was removed by centrifugation at 15000 g for 15 min at 4 °C. Total cleared lysates were subjected to SDS/PAGE (5% gels), transferred to nitrocellulose and immunodetected with specific mAbs against each type of IP₃R.

[Ca²⁺]_i measurements in cell suspension

[Ca²⁺]_i was measured using Fura-2. Either thymocytes or splenocytes (1×10^7 /ml) were loaded with 5 μ M Fura-2 AM (Molecular Probes, Eugene, OR, U.S.A.) in RPMI 1640 medium [(2% (v/v) fetal-calf serum) containing 2 μ M 2-mercaptoethanol. After incubation for 30 min at 37 °C, the cells were washed and resuspended in RPMI medium [10% (v/v) fetal-calf serum]. Before [Ca²⁺]_i measurement the cells were washed twice and then resuspended in a modified Krebs-Ringer solution [125 mM NaCl/5 mM KCl/1 mM MgSO₄/1 mM Na₃PO₄/1 mM CaCl₂/5.5 mM glucose/20 mM Hepes/KOH (pH 7.2)] to give a cell concentration of 4×10^6 cell/ml. The fluorescence of the stirred cell suspension was measured by using CAF-110 (Jasco, Tokyo, Japan) at 30 °C with $\lambda_{\text{excitation}}$ 340 nm and 380 nm and $\lambda_{\text{emission}}$ 510 nm. The [Ca²⁺]_i was calculated as described previously [13].

Immunofluorescence analysis

mAbs used were PE-labelled anti-Thy1.2, PE-labelled anti-CD4, FITC-labelled anti-CD8 and FITC-labelled anti-B220. After the staining, the cells were suspended in buffer containing 1 μ g/ml 7-aminoactinomycin D (7AAD, Sigma) to exclude dead cells from the analysis. Stained cells were analysed on a FACscan (Becton Dickinson, Mountain View, CA, U.S.A.). Only cells in the lymphocyte gate (as defined according to side and forward scatters) were analysed.

Cell culture and proliferation assay

Cells were cultured in RPMI 1640 medium [10% (v/v) fetal-calf serum] supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 μ M 2-mercaptoethanol. For proliferation assays, cells were cultured at 2×10^5 cells/well in a 96-well flat-bottom plate with concanavalin A (5 μ g/ml) or various concentrations of immobilized anti-CD3 mAb. The cells were pulse-labelled with [³H]thymidine (0.2 μ Ci/well) during the last 8 h of a 72 h culture period and [³H]thymidine incorporated was measured by a MATRIX 96 Direct β -Counter (Hewlett-Packard, Boise, ID, U.S.A.). All assays were done in triplicate.

RESULTS AND DISCUSSION

IP₃R1^{-/-} mice with a pure C57BL/6 genetic background showed the same phenotype as that of F2 mice, which included ataxia, epileptic seizures, a small body size and death before weaning [10]. To investigate a loss of function of IP₃R1 and to clarify the significance of IP₃R1 in T-cells, we needed to eliminate any effects resulting from undernourishment and stress by the severe phenotypic changes on the immune system. Therefore, we transplanted the BM cells of either IP₃R1^{+/+} (control) or IP₃R1^{-/-} mice into irradiated wild-type mice and studied development and function of T-cells from the recipient mice. Both recipient mice transplanted with the IP₃R1^{+/+} BM cells and the IP₃R1^{-/-} ones grew normally and no differences in the phenotypic appearance was observed.

To confirm whether the donor IP₃R1^{-/-} BM cells replaced the original IP₃R1^{+/+} BM cells, the recipient thymocytes and splenocytes were subjected to Western blot analysis with the

Figure 1 Western blot analysis of thymocytes and splenocytes lacking IP₃R1

Western blot analysis of total cell lysates of thymocytes (thy) and splenocytes (spl) (1×10^6 cells/lane) from the recipient mice transplanted with IP₃R1 +/+ or IP₃R1 -/- BM cells with specific mAbs against IP₃R1, IP₃R2 and IP₃R3. Molecular mass markers are indicated on the left.

specific mAb against IP₃R1, 18A10 (Figure 1). The IP₃R1 was not detectable in thymocytes of the recipient mice; however, a faint but negligible band of IP₃R1 was detectable in splenocytes, which was estimated to be $0.88 \pm 0.62\%$ (mean $\pm$ S.D., $n = 3$) of the control splenocytes by densitometric analysis using NIH Image 1.58 (National Institutes of Health, Bethesda, MD, U.S.A.), indicating that the recipient original IP₃R1 +/+ BM cells were replaced by the donor IP₃R1 -/- BM cells after transplantation. This residual, but negligible, expression of IP₃R1 was possibly derived from the residual recipient IP₃R1 +/+ lymphocytes or from contamination of other tissues. The expression of IP₃R2 and IP₃R3 was also analysed by Western blotting with the specific mAbs KM1083 and KM1082 for IP₃R2 and IP₃R3 respectively (Figure 1). In contrast, the expression levels of both IP₃R2 and IP₃R3 were unaltered, suggesting that no significant compensation for loss of IP₃R1 by IP₃R2 and IP₃R3 occurred.

As demonstrated in the Jurkat cells lacking IP₃R1, if the IP₃R1 is essential for TCR signalling and if the IP₃R1 deficiency results in resistance to apoptosis [8,9], IP₃R1 -/- T-cells would show an abnormal phenotype, e.g., neither $[Ca^{2+}]_i$ increases nor cell proliferation would be observed after TCR stimulation. In addition, apoptosis resistance would result in loss of negative selection, thereby causing proportions of the T-cell phenotype in the thymus to be abnormal. We investigated the properties of T-cells *in vivo* by immunofluorescence analysis. The cell number of the IP₃R1 -/- thymocytes and splenocytes were almost the same as those of the control (Table 1). Quantification of the CD4+ and CD8+ populations using immunofluorescence analysis revealed that, in both thymocytes and splenocytes, these T-cell populations were unaltered, suggesting that the IP₃R1 -/- T-cells differentiate normally and that IP₃R1 deficiency does not result in the failure of apoptosis during negative selection (Table 1).

We then investigated the function of IP₃R1 in primary T-cells. We examined the proliferative response to TCR stimulation. Table 2 summarizes $[^3H]$ thymidine incorporation upon TCR

Table 1 Immunofluorescence analysis of thymocytes and splenocytes

Results were expressed as mean percentages $\pm$ S.D. ($n = 5$).

Property	Genetic phenotype	
	IP ₃ R1 +/+	IP ₃ R1 -/-
Cell number		
Thymocyte $\times 10^7$	9.40 ± 3.24	10.3 ± 3.46
Splenocyte $\times 10^7$	11.5 ± 1.46	9.62 ± 2.37
Thymocyte phenotype		
DN %	2.42 ± 0.721	2.39 ± 0.954
DP %	85.4 ± 3.98	83.6 ± 2.70
SP(CD4+) %	8.97 ± 3.35	10.3 ± 1.05
SP(CD8+) %	3.18 ± 1.04	3.72 ± 1.19
Splenocyte phenotype		
B220+ (B-cell) %	63.2 ± 4.02	64.1 ± 5.92
Thy1+ (T-cell) %	27.8 ± 6.12	22.3 ± 5.62
Splenic T-cell phenotype		
CD4+CD8- %	22.0 ± 3.53	18.1 ± 2.59
CD4-CD8+ %	8.70 ± 1.25	7.04 ± 2.21
Ratio (CD4+/CD8+)	2.53	2.57

stimuli by anti-CD3 mAb. The IP₃R1-deficient T-cells in both thymocytes and splenocytes showed normal proliferative responses to stimulation *in vitro*, indicating that the IP₃R1 is not essential for proliferation of T-cells via TCRs. The cell viability of primary cells was also examined. The cells from IP₃R1 -/- recipient mice were cultured for 24 h in culture medium, and then the percentage of viable cells was determined on a FACScan after staining with 7AAD. There was no difference between the cell viability of IP₃R1 -/- T-cells and that of the control (results not shown). This result suggests that IP₃R1 is not an essential mediator of the apoptotic signal in primary T-cells.

Figure 2(A) shows typical profiles of $[Ca^{2+}]_i$ increases after TCR stimulation with the anti-CD3 mAb 2C11. In contrast with the Jurkat cells lacking IP₃R1 [9], both thymocytes and splenocytes lacking IP₃R1 were capable of increasing $[Ca^{2+}]_i$ after stimulation with anti-CD3 mAb in a similar manner to the control cells (Figure 2A). In Ca^{2+} -free solution, the initial rapid $[Ca^{2+}]_i$ increase, but not the subsequent sustained $[Ca^{2+}]_i$ influx, was observed (results not shown), confirming the previous observations that the initial $[Ca^{2+}]_i$ increase is due to Ca^{2+} release from the intracellular store and the subsequent sustained $[Ca^{2+}]_i$ increase is due to Ca^{2+} influx from extracellular medium. The maximal $[Ca^{2+}]_i$ increases ($n = 5$) are summarized in Figure 2(B) ($n = 5$). There were no differences between IP₃R1 +/+ and IP₃R1 -/-, suggesting that a loss of IP₃R1 function did not affect Ca^{2+} mobilization after TCR stimulation. We also examined $[Ca^{2+}]_i$ increases after TCR stimulation in the thymocytes and splenocytes from 17-day-old IP₃R1 -/- mice (C57BL/6). Stimulation of the IP₃R1 -/- thymocytes and splenocytes with anti-CD3 mAb also resulted in the same responses (results not shown) and immunofluorescence analysis also revealed that, in both thymocytes and splenocytes, these T-cells differentiate normally (results not shown), confirming that our results obtained using the transplanted mice reflect a loss of IP₃R1 function in T-cells. These results indicate that IP₃R1 is not essential for either Ca^{2+} mobilization after TCR stimulation or cell proliferation and thus the remaining types of IP₃R, IP₃R2 and IP₃R3, are sufficient for Ca^{2+} mobilization and cell proliferation.

The discrepancy between the results reported for Jurkat cells

Table 2 Proliferative response of thymocytes and splenocytes to anti-CD3 mAb

Thymocytes and splenocytes were examined for proliferative response to the indicated stimuli. Results were expressed as mean c.p.m.±S.D. of triplicate wells. Representative results of three independent experiments are shown.

Thymocytes					
Stimulant...	None	Immobilized anti-CD3			Concanavalin A (5 µg/ml)
		0.4 µg/ml	2.0 µg/ml	10 µg/ml	
IP3R1 +/+	27 ± 7	1680 ± 610	4260 ± 200	8620 ± 1650	12300 ± 2100
IP3R1 -/-	59 ± 17	1240 ± 670	5260 ± 600	7420 ± 1480	10900 ± 1800
Splenocytes					
Stimulant...	None	Immobilized anti-CD3			Concanavalin A (5 µg/ml)
		0.016 µg/ml	0.08 µg/ml	0.4 µg/ml	
IP3R1 +/+	175 ± 37	26000 ± 2000	32800 ± 1800	34000 ± 800	41400 ± 2300
IP3R1 -/-	348 ± 154	26200 ± 3700	31700 ± 1100	34300 ± 1600	38400 ± 3300

Figure 2 TCR stimulation with anti-CD3 mAb-induced $[Ca^{2+}]_i$ increase in the T-cells lacking IP₃R1

(A) The IP₃R1 +/+ and IP₃R1 -/- thymocytes and splenocytes were stimulated with anti-CD3 mAb 2C11 (2.5 µg/ml) at *t* = 100 s. Anti-CD3 mAb induced an initial $[Ca^{2+}]_i$ increase, followed by a sustained $[Ca^{2+}]_i$ increase, in all cases. **(B)** The $[Ca^{2+}]_i$ responses on TCR stimulation with anti-CD3 mAb are summarized. Error bars represent means±S.D. (*n* = 5). thy, thymocytes; spl, splenocytes.

lacking IP₃R1 [8,9] and this study using IP₃R1 -/- T-cells might be due to the different cell-types used. In this case, the IP₃R1 might be the dominant type of IP₃R in the Jurkat cells and the expression of IP₃R2 and IP₃R3 might be too low to function as the Ca²⁺-mobilizing channels in TCR signalling. Alternatively, the discrepancy may ensue from the different preparation of IP₃R1-deficient T-cells. In this study, we produced T-cells lacking IP₃R1 using gene targeting and transplantation, in which the IP₃R1 gene was specifically disrupted and this disruption did not affect the expression of IP₃R2 and IP₃R3. Therefore we could evaluate the significance of IP₃R1 in TCR signalling *in vivo* and *in vitro*. In contrast, Jurkat cells lacking IP₃R1 were established by the stable expression of an antisense cDNA construct of IP₃R1 to prevent the efficient translation of IP₃R1 mRNA. Using this strategy, the authors selected the 5'-region of 2.9 kb (nt -247 to -2592) in the human IP₃R1 cDNA for the antisense construct, which has high homology with the IP₃R2 and IP₃R3 (60–75 %) [8,14–16]. Therefore it is possible that the antisense construct of IP₃R1 inhibited not only IP₃R1 expression, but also IP₃R2 and IP₃R3 expression, resulting in the observed failure to mobilize Ca²⁺ from intracellular Ca²⁺ stores after TCR stimulation. In fact the expression of IP₃R2 and IP₃R3 in the IP₃R1-deficient Jurkat cells was detectable, but reduced [8]. If this is the case, it is possible to conclude that only the IP₃R-1, -2 and -3 triple-deficient T-cells result in loss of Ca²⁺ mobilization after TCR activation, and that Ca²⁺ released via not only IP₃R1 but also IP₃R2 and IP₃R3 might be essential for TCR signalling and a critical mediator of apoptosis. Recently, Sugawara et al. [17] demonstrated that DT40 cells (a chick B-cell line) in which a single type of IP₃R has been deleted by gene targeting mobilize Ca²⁺ in response to B-cell antigen receptor (BCR) stimulation, whereas this Ca²⁺ mobilization is abolished in B-cells lacking all three types of IP₃R and the BCR-induced apoptosis is inhibited only in triple-deficient cells. We also observed that the IP₃R1 -/- splenocytes showed normal Ca²⁺ mobilization, normal cell proliferation after BCR stimulation with the anti-(mouse IgM) and normal cell viability (results not shown). Therefore the observations of Sugawara et al. and ourselves strongly suggest that not only IP₃R1 but also IP₃R2 and IP₃R3 are essential for BCR or TCR signalling and apoptosis. A third possibility for the discrepancy is that the antisense cDNA construct of IP₃R1 could

cause additional side effects on the expression of other proteins involved in TCR signalling.

In conclusion, we have analysed a loss of function of the IP₃R1 in T-cells in terms of T-cell development, Ca²⁺ mobilization and cell proliferation after TCR stimulation and cell viability. In clear contrast with the previously reported observations in Jurkat cells lacking IP₃R1 [8,9], we have observed no significant differences in several experiments between IP₃R1+/+ and IP₃R1−/− T-cells, i.e. IP₃R1−/− T-cells were capable of mobilizing Ca²⁺ after TCR stimulation and showed normal cell differentiation, proliferation and viability. These results indicate that IP₃R1 is not the sole receptor responsible for either Ca²⁺ mobilization after TCR activation or for cell proliferation, and that the remaining IP₃R2 and IP₃R3 are sufficient for T-cell development and function even in the absence of IP₃R1.

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