Ionotropic 5-hydroxytryptamine type 3 receptor activates the protein kinase C-dependent phospholipase D pathway in human T-cells

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The present study was undertaken to investigate the role of the 5-hydroxytryptamine (5-HT) ionotropic receptor 5-HT₃ in the activation of human Jurkat T-cells. 5-HT and 2-methyl-5-HT (2Me-5-HT), an agonist of the 5-HT₃ receptor, induced increases in intracellular free Na⁺ concentrations, [Na⁺]_i, via opening of the ionotropic receptor in these cells. These two serotonergic (5-hydroxytryptaminergic) agents potentiated phytohaemagglutinin (PHA)-induced T-cell activation. However, they failed to potentiate dioctanoglycerol-plus-ionomycin-stimulated T-cell blastogenesis. Interestingly, an inhibitor of protein kinase C (PKC), GF 109203X, curtailed significantly 5-HT and 2Me-5-HT-potentiated T-cell activation. These results demonstrate that the opening of the 5-HT₃ ionotropic receptor is implicated in T-cell activation via the PKC pathway. Furthermore, 5-HT and

2Me-5-HT stimulated phospholipase D (PLD) activity, as measured by the production of phosphatidylethanol and phosphatidylbutanol at the expense of phosphatidic acid (PA). GF 109203X significantly curtailed the 5-HT- and 2Me-5-HT-induced PLD activity and T-cell activation. The PLD/PA pathway stimulated by these two serotonergic agents resulted in the production of 1,2-diacylglycerol (DAG) mass in Jurkat T-cells. These results altogether suggest that 5-HT and 2Me-5-HT potentiate T-cell activation via increases in [Na⁺]_i and the activation of the PKC-dependent PLD pathway.

Key words: 1,2-diacylglycerol, 5-hydroxy-2-methyltryptamine, Jurkat T-cells, Na⁺ influx, phospholipase D.

INTRODUCTION

5-Hydroxytryptamine (5-HT, 'serotonin') has been shown to influence the immune system of mammals as well as of other vertebrates [1–4]. The effects of 5-HT have been found to differ according to the concentration and the cell types used. *In vitro*, addition of exogenous 5-HT has been found to suppress phytohaemagglutinin (PHA)-induced blastogenesis [5]. 5-HT has been found to augment interferon-γ-induced phagocytosis [6] and natural killer cytotoxicity of human CD16⁺/non-T-lymphocytes [7]. Recently there has been an upsurge of information indicating that 5-HT can stimulate the immune system via lymphocyte membrane receptors [3,8–11]. As far as the multiplicity of serotonergic receptors is concerned [12], it is considered that the 5-HT₁ class consists of at least five subtypes, namely 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}, which are coupled to G-proteins. The 5-HT₂ class is subdivided into three subtypes, namely 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}, which are coupled to inositol metabolism. Besides 5-HT₄, 5-HT₅ and 5-HT₆ receptors, there is the 5-HT₃ receptor, which is an ionotropic receptor, permeant to cations with a high selectivity to Na⁺ inward movements [12].

It has been shown that 5-HT up-regulates, in a dose-dependent manner, mitogen-stimulated B-lymphocyte proliferation via 5-HT_{1A} receptors present on these cells [8]. Hence 5-HT and the agonists of 5-HT_{1A} receptors promote the transition of murine B-cells from G₀/G₁-phase to the S-phase of the cell cycle [8]. Jurkat T-cells and mitogen-activated, but not resting, human T-cells also express the 5-HT_{1A} receptor subtype which is coupled to

elevation of intracellular free calcium and inositol metabolism in these cells [9]. The T-cell 5-HT_{1A} receptor subtype also modulates the production of interleukin-2 and interferon-γ [10]. Those workers have also demonstrated that *de novo* synthesis of 5-HT in T lymphocytes is necessary for the T-cell responses *in vitro* and *in vivo* [9,10]. By employing various agonists and antagonists, Choquet and Korn [13] have shown that 5-HT also regulates the voltage-gated potassium channels via 5-HT₁-like and 5-HT₃ receptors in a murine pre-B-cell line. Though there are a number of studies available on the role of Na⁺/K⁺ movements in T-cell activation [14], not much is known about the 5-HT₃ ionotropic receptor in these lymphocytes, except a few recent reports in which its presence has been demonstrated in T-cells [11,13,15,16]. We have also characterized the 5-HT₃ ionotropic receptor in Jurkat T-cells, and we have observed that 5-HT stimulated PHA-induced proliferation of these cells by promoting the transition from S-phase to G₂/M phase of the T-cell cycle [16]. In the present study we have further elucidated the mechanisms of T-cell activation via the 5-HT₃ ionotropic receptor in Jurkat T-cells.

MATERIALS AND METHODS

Materials

Sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI/AM) was procured from Molecular Probes, Eugene, OR, U.S.A. 2Me-5-HT, metoclopramide and BRL-43694 were purchased from Research Biochemicals, Natick, MA, U.S.A. All

Abbreviations used: DAG, 1,2-diacylglycerol; DiC₈, dioctanoglycerol; 5-HT, 5-hydroxytryptamine (serotonin); 2Me-5-HT, 5-hydroxy-2-methyltryptamine; MCP, metoclopramide; PA, phosphatidic acid; PC, phosphatidylcholine; PBuT, phosphatidylbutanol; PET, phosphatidylethanol; PHA, phytohaemagglutinin; PLD, phospholipase D; SBFI/AM, sodium-binding benzofuran isophthalate acetoxymethyl ester; [Na⁺]_i, intracellular Na⁺.

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other chemicals were obtained from Sigma. [^3H]Thymidine (sp. radioactivity 20 Ci/mmol) was purchased from Amersham Radiochemicals. [^3H]Oleic acid and [^{32}P]ATP (sp. radioactivity 3000 Ci/mmol) were from NEN–Du Pont. 1,2-Diacylglycerol (DAG) kinase (from *Escherichia coli*) and the PKC inhibitor, GF 109203X, a compound of the bisindolylmaleimide family, were obtained from Calbiochem. Silica-gel 60 CF254 plates were from Merck.

Human (Jurkat) T-cells were cultured in RPMI-1640 medium supplemented with L-glutamine and 10% (v/v) foetal-calf serum at 37 °C in a humidified chamber containing air/CO₂ (19:1). Cell viability was assessed by the Trypan Blue exclusion test. Cell numbers were determined by using a haemocytometer.

Calibration of SBFI/AM fluorescence

The probe SBFI/AM was used to measure increases in free Na⁺ concentrations. The cells (4×10^6 /ml) were loaded at 5 μM of the probe for 60 min at 37 °C in loading buffer which contained the following (in mM): NaCl, 140; KCl, 5; MgCl₂, 1; sodium Hepes, 20; Na₂HPO₄, 1; glucose, 10 (pH 7.4). After loading, the cells were washed three times (2000 g for 10 min) with the same buffer. *In situ* calibration was performed as described by Harotunian et al. [17] by using appropriate mixtures of high-Na⁺ and high-K⁺ buffers. The high-Na⁺ buffer contained the following (in mM): sodium gluconate, 110; NaCl, 130; MgCl₂, 0.6; sodium Hepes, 10; EGTA, 1 (pH 7.4). The high-K⁺ buffer was identical except for complete replacement of Na⁺ by K⁺. A mixture (10 μg /ml) of antibiotics (gramicidin D, nigericin and monensin) was used to calibrate the Na⁺ concentrations. Before recording the Na⁺ movements, amiloride was added into the cuvette. The cells were continuously stirred throughout the experimentation. The fluorescence intensities were measured in a PTI (Photon Technology International) spectrofluorimeter in ratio mode at 340 and 360 nm (excitation filters) and 480 nm (emission filters). The intracellular Na⁺ ([Na⁺]_i), contents were calculated by using the following equation:

$$[\text{Na}^+]_i = K_d \times \beta \times (R - R_{\min}) / (R_{\max} - R)$$

K_d and β represent respectively the dissociation constant and the ratio of excitation efficiencies of free to Na⁺-bound SBFI at 380 nm. R stands for the ratio of fluorescence intensities at 340 and 360 nm excitation, whereas $R_{\min}$ and $R_{\max}$ represent respectively the ratios at 0 mM Na⁺ and at saturating Na⁺ concentration (50 mM). A value of 19 mM for K_d was added into the calculations.

T-cell proliferation assay

The human (Jurkat) T-cells (50×10^3 /assay) were cultured in 96-well flat-bottom tissue-culture plates (Nunc) in the presence of PHA (0.5 μg /ml). Cells were distributed in triplicate as follows: 140 μl , cell suspension; 20 μl , mitogen or medium; and 20 μl , 2-Me-5HT (as indicated in the legends) or test molecules or 20 μl of medium. Plates were incubated at 37 ± 2 °C (90% humidity). After 72 h, 20 μl of [^3H]thymidine (20 Ci/mmol, 0.8 μCi /well) were added and, 12 h later, the cells were harvested using an automatic cell harvester (Basic 96 Harvester, Skatron), trapping their DNA on to glass filter mats. Dried filter circles were placed in plastic Minivials (Packard), 3.5 ml of Opti-fluor-O (Packard) was added and the radioactivity was recorded in a liquid-scintillation counter (Packard).

Determination of phospholipase D (PLD) activity

The PLD activity was determined by measuring the production of phosphatidylethanol (PET) or phosphatidylbutanol (PBut) when the cells were cultured respectively in the presence of ethanol and butan-1-ol [18]. The Jurkat T-cells (10×10^6 cells/ml) were incubated with 5 μCi /ml [^3H]oleic acid for 24 h in order to label the phospholipids. The cells were washed and preincubated with fresh medium for 30 min. Ethanol (1%) or butan-1-ol (0.05%) was present in the culture medium during the experimentation. The lipids were extracted as follows. After washing the cells with ice-cold PBS, pH 7.4, the cells were solubilized with 0.5 ml of 0.2% SDS for 10 min, and chloroform and 0.2 M NaCl were added to yield the final chloroform/methanol/water proportions 1:1:0.9 (by vol.). After centrifugation, the lower organic phase was separated from the upper phase and dried under nitrogen. Lipids were dissolved in chloroform and kept at -20 °C until analysis.

DAG kinase assay

Production of DAG was assessed by the DAG kinase assay as described elsewhere [18]. In brief, the lipids containing DAG were solubilized in an octyl β -glucoside/cardiophilin solution and incubated with the DAG kinase from *E. coli*, in the presence of [γ - ^{32}P]ATP to convert the DAG into [^{32}P]phosphatidic acid ([^{32}P]PA). The lipids were extracted as described above and the PA was separated by TLC, using the solvent chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol.). Authentic DAG was run in parallel. The corresponding spots were identified and scraped off from the TLC plates. The radioactivity was counted in a liquid-scintillation counter.

Statistical analysis

Results are shown as means $\pm$ S.D. for quadruplicate assay samples, reproduced independently at least three times. Statistical analysis of the data was carried out using STATISTICA (Version 4.1, 1994; Statsoft, Paris, France). The significance of the differences between mean values was determined by analysis of variance one way, followed by a least-significant-difference test.

RESULTS

5-HT and 2Me-5-HT induce increases in [Na⁺]_i

In SBFI/AM-loaded Jurkat T-cells, 5-HT stimulated the influx of Na⁺. 2Me-5-HT, an agonist of the 5-HT₃ ionotropic receptor, also induced increases in [Na⁺]_i (Figure 1). The EC₅₀ values for 5-HT and 2Me-5-HT required to induce the increases in [Na⁺]_i were calculated to be respectively 1.5 and 1.75 μM . The increase in [Na⁺]_i induced by 2Me-5-HT was curtailed significantly by the addition of metoclopramide (MCP), an antagonist of the 5-HT₃ ionotropic receptor in these cells (Figure 1). The IC₅₀ values for the 5-HT₃ receptor antagonists, MCP and BRL-43694, on the inhibition of increases in [Na⁺]_i were found to be respectively 2.5 and 3 μM . Interestingly, spiperone and ketanserin, respective antagonists of 5-HT₁ and 5-HT₂ receptor subtypes, failed to diminish the 2Me-5-HT-induced-increases in [Na⁺]_i in these cells (Figure 1).

5-HT and 2Me-5-HT potentiate the PHA-stimulated, but not DiC₈- and-ionomycin-stimulated, T-cell proliferation

5-HT and 2Me-5-HT potentiated the PHA-induced proliferation of Jurkat T-cells (Figure 2). Addition of MCP, an antagonist of the 5-HT₃ ionotropic receptor, abolished the stimulatory effects

Figure 1 Na⁺ influx via the 5-HT₃ ionotropic receptor in human Jurkat T-cells

The Jurkat T-cells (4×10^6 cells/assay) were loaded with the fluorescent probe SBFI/AM as described in Materials and methods section. Arrowheads indicate the times when the test compounds were added. 2-Me-5-HT ('2-methyl 5HT') ($5 \mu\text{M}$), 5-HT ('5HT') ($5 \mu\text{M}$), MCP ($10 \mu\text{M}$), spiperone ($5 \mu\text{M}$) and ketanserin ($5 \mu\text{M}$) were added into the cuvettes without any interruptions in the recordings. The Figure shows the identical traces reproduced several times independently.

of these two serotonergic agents on T-cell activation. Spiperone and ketanserin did not diminish the T-cell proliferation induced by 2Me-5-HT (Figure 2). In order to probe PKC activation via the 5-HT₃ ionotropic receptor, T-cells were also stimulated by DiC₈ plus ionomycin. Hence we observed that neither 5-HT nor 2Me-5-HT potentiated T-cell activation induced by DiC₈ plus ionomycin. Furthermore, a potent PKC inhibitor, GF 109203X, curtailed significantly the stimulatory actions of both 5-HT and 2Me-5-HT on DiC₈-plus-ionomycin- and PHA-induced T-cell blastogenesis (Figure 2).

5-HT and 2Me-5-HT stimulate the PKC-dependent PLD activity

In order to detect the PLD activity, we measured production of PET and PBuT via transphosphatidylolation of phosphatidylcholine (PC) in the presence of the respective primary alcohols, ethanol and n-butanol. Hence, the T-cells produce PET and PBuT at the expense DAG [19]. In Jurkat T-cells, stimulated in the presence of ethanol, 5-HT and 2Me-5-HT both induced the production of PET in a time-dependent manner (Figure 3). PET production was observed even after 5 min of incubation with these serotonergic agents. Treatment with PHA alone did not induce significantly PET production (results not shown). 5-HT and 2Me-5-HT also induced production of PBuT in these cells

(Table 1). MCP, an antagonist of the 5-HT₃ ionotropic receptor, abolished PET and PBuT production induced by 5-HT and 2Me-5-HT (Figure 4 and Table 1). The antagonists of 5-HT₁ and 5-HT₂ receptor subtypes (spiperone and ketanserin) failed to curtail the 5-HT and 2Me-5-HT-induced PET production in these cells (Figure 4). GF 109203X, a PKC inhibitor, abolished the stimulatory effects of 5-HT and 2Me-5-HT on PET production in Jurkat T-cells (Figure 4).

5-HT and 2Me-5-HT induce the DAG production via the PLD pathway

In the DAG kinase assays we observed that 5-HT and 2Me-5-HT stimulated the production of DAG in a dose-dependent manner (Figure 5). In fact, under the action of PLD, the hydrolysis of PC gives rise to PA, which is then converted into DAG by PA phosphohydrolase. In order to evaluate the implication of the PLD/PA pathway in DAG production, we used propanolol, an inhibitor of PA phosphohydrolase. Hence we observed that the addition of propanolol ($100 \mu\text{M}$) to 2Me-5-HT-treated Jurkat T-cells diminished the production of DAG concomitantly with an increase in PA levels, i.e., the DAG mass was reduced from 0.5 ± 0.01 nmol to 0.1 ± 0.009 nmol/assay and the PA levels were increased from 50 ± 10 c.p.m. (control) to 2150 ± 146 c.p.m.

Figure 2 Effects of 5-HT and 2Me-5-HT on PHA- and DiC₈-plus-ionomycin-stimulated proliferation of human Jurkat T-cells

The human Jurkat T-cells (50×10^3 cells/assay) were cultured in 96-well flat-bottom tissue-culture plates in the presence of different test compounds and the proliferation assays were performed as described in the Materials and methods section. The test agents were added at the following concentrations: PHA, 0.5 μ g/ml; 5-HT, 5 μ M; 2Me-5-HT, 5 μ M; MCP, 10 μ M; spiperone, 5 μ M; ketanserin, 5 μ M; DiC₈, 50 μ M, plus ionomycin ('IONO'), 0.5 μ M; GF 109203X, 500 nM. The asterisks (*) demonstrate that the values are significant ($P < 0.001$) when compared with control values.

DISCUSSION

In the present study we have observed that 5-HT and 2Me-5-HT, an agonist of the 5-HT₃ ionotropic receptor, evoked increases in $[Na^+]_i$. These increases in $[Na^+]_i$ were abolished by MCP and BRL-43694, whereas spiperone and ketanserin, the respective antagonists of 5-HT₁ and 5-HT₂ receptor subtypes, failed to abolish the action of 2Me-5-HT. Furthermore, 5-HT and 2Me-5-HT potentiated the T-cell proliferation induced by PHA. The mechanisms of action of these two agents were abolished by MCP. These observations corroborate our recent study in which we have reported the presence of functional 5-HT₃ ionotropic receptor in Jurkat T-cells [16]. We also activated T-cells directly by the addition of DiC₈ and ionomycin in order to by-pass the implication of the T-cell receptor in T-cell activation and to elucidate the role of PKC in 2Me-5-HT-induced T-cell proliferation. DiC₈ and ionomycin have been used by several investigators to activate directly PKC and T-cell proliferation [20,21]. Interestingly, the two serotonergic agents failed to potentiate T-cell stimulation initiated by DiC₈ and ionomycin. Addition of GF 109203X, a PKC inhibitor of the bisindolylmaleimide family, inhibited both the DiC₈ and ionomycin, and 5-HT and 2Me-5-HT potentiated PHA-stimulated T-cell proliferation. These results demonstrate that the agonists of the

Table 1 Effects of serotonergic agents on PBuT production in Jurkat T-cells

For further details, see the Materials and methods section.

Agent(s)	PBuT production (c.p.m./assay)
Control	150 ± 25
5-HT	6000 ± 124
2Me-5-HT	6600 ± 160
5-HT + MCP	200 ± 40
2Me-5-HT + MCP	175 ± 55

Figure 3 Production of PET in response to 5-HT (●) and 2Me-5-HT (□) in human Jurkat T-cells

The human Jurkat T-cells were prelabelled overnight with [³H]oleic acid (5 μ Ci/ml) and PET production was measured as described in the Materials and methods section.

Figure 4 Inhibition of PET production by various agents in human Jurkat T-cells

The human Jurkat T-cells were prelabelled with [³H]oleic acid (5 μ Ci/ml) overnight and PET production was measured as described in the Materials and methods section. The agents were added at different concentrations: 5-HT ('5HT'), 5 μ M; 2Me-5-HT ('2M-5HT'), 5 μ M; MCP, 10 μ M; spiperone, 5 μ M; ketanserin, 5 μ M; GF 109203X, 500 nM. The incubation period in the presence of serotonergic agonists was 10 min. The asterisks demonstrate that the values are significant ($P < 0.001$) as compared with the control (*), the 5-HT (**) group and the 2Me-5-HT (***) group.

Figure 5 Dose-dependent production of 1,2-DAG in response to 5-HT (●) and 2Me-5-HT (□) in human Jurkat T-cells

The human Jurkat T-cells (10×10^6 cells/assay) were incubated with increasing concentrations (0–1000 nM) of 5-HT or 2Me-5-HT for 10 min. The lipids were extracted and the DAG mass was measured by the DAG kinase assay described in the Materials and methods section.

5-HT₃ ionotropic receptor potentiate T-cell proliferation via activation of the PKC.

How does the 5-HT₃ ionotropic receptor potentiate the T-cell stimulation via PKC? We can hypothesize that 5-HT and 2Me-5-HT-induced increases in $[Na^+]_i$ via the 5-HT₃ ionotropic receptor may activate the phospholipase C/PKC pathway or other phosphorylating signalling proteins, like tyrosine kinase, coupled to T-cell receptor [22]. Rozengurt and Courtenay [23] have shown that increasing $[Na^+]_i$ synergizes with insulin and epidermal growth factor to elicit DNA synthesis. Similarly, Rosoff and Cantley [24] noticed that increasing $[Na^+]_i$ directly with monensin or ouabain elicited differentiation in a pre-B-cell line. Gierschik et al. [25] have reported that an increase in $[Na^+]_i$ regulates the G-protein-mediated signal by ions in cardiomyocytes. Similarly, Borin and Stiffert [26] have observed that an increase in $[Na^+]_i$ influences the coupling of the thrombin receptor to G-protein in human platelets, being a key step in the regulation of receptor-mediated feedback mechanisms. Whether Na^+ can activate protein kinase C or these cells possess a Na^+ -dependent phospholipase C remains to be ascertained in future. However, in our study, the entry of Na^+ may be considered as a 'co-stimulatory' signal which may by-pass the known co-stimulatory pathways that involve CD28 protein on T-cells and B7.1 on antigen-presenting cells. This notion can be supported by the observations of Rhodes et al. [28], who have recently shown that the immunostimulatory effects of Schiff-base-forming molecules are mediated by a co-stimulatory signal, elicited by clofilium-sensitive Na^+ influx, leading to phosphorylation of key signalling proteins.

In the present study, the sole target of 5-HT and 2Me-5-HT is not only PKC but also the PKC-dependent PLD activity, measured by the production of PET and PBuT. In fact, the PC-specific PLD can give rise to PET and PBuT via transphosphatidylolation in the presence of primary alcohols, at the expense of DAG production. We observed that 5-HT and 2Me-5-HT induced the production of PET and PBuT and their levels were diminished when the cells were treated simultaneously with MCP, an antagonist of the 5-HT₃ ionotropic receptor. The PET and PBuT production induced by ethanol and butan-1-ol was very low as compared with that induced by serotonergic agonists. These observations suggest that the functioning of the 5-HT₃ ionotropic receptor is implicated in the activation of the PLD in Jurkat T-cells. Furthermore, this PLD activity induced by the

serotonergic agents was PKC-dependent, since GF 109203X curtailed this stimulated activity in Jurkat T-cells. These results contribute to the findings of several workers who have characterized the PKC-dependent PLD activity in different cell lines [19,29–31]. Cook and Wakelam [29] observed that bombesin activated PLD via a mechanism involving PKC activation in 3T3 cells. Similarly, a PKC-dependent PLD was found to catalyse the synthesis of PET in neuroblastoma hybrid cells [30]. In another study conducted on human neutrophils, Lopez et al. [31] observed that staurosporin inhibited PLD activity in these cells.

However, it seems likely that the PKC-dependent PLD, once activated by 5-HT and 2Me-5-HT, may produce PA and, later on, DAG. To probe this notion, of whether the PLD/PA pathway, stimulated by the serotonergic agents, contributes to the production of DAG in Jurkat T-cells, we conducted further experiments. Hence, we employed propranolol, an inhibitor of PA phosphohydrolase. We should recall that the phosphohydrolase dephosphorylates PA into DAG, and the inhibition of its activity by propranolol will result in increased PA levels and decreased DAG contents in treated cells. As expected, we observed that 5-HT and 2Me-5-HT produced DAG, and the addition of propranolol to 2Me-5-HT-treated cells increased the PA levels and diminished the DAG mass in Jurkat T-cells. These observations on the activation of PLD by serotonergic agents corroborate the findings of Kurscheid-Reich and co-workers [32,33], who have found that, in mesangial cells, 5-HT activates the PC-dependent PLD/PA pathway which contributed to DAG production responsible for the mitogenic action of 5-HT. However, these workers did not mention which serotonergic receptor was involved in PLD activation in these cells. It is also noteworthy that, in our study, PHA did not induce an increase in PET content in these cells (results not shown).

During the course of T-cell activation, the early events are transient signals which comprise PKC activation, a rise in calcium concentration and expression of immediate early genes, whereas the late events required for the sustained activation, involve prolonged activation of PKC, which is under the regulation of PLD and phospholipase A₂ [27]. The induction of PKC-dependent PLD activity by 5-HT and 2Me-5-HT in these cells indicates that the PA produced by PLD via the 5-HT₃ ionotropic receptor, coupled to Na^+ influx, may give rise to the endogenous DAG which can assure the prolonged activation of Jurkat T-cells.

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