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The Effect of the JNK Inhibitor, JIP Peptide, on Human T Lymphocyte Proliferation and Cytokine Production¹

Michelle Melino,* Hussan S. Hii, †‡ Shaun R. McColl,* and Antonio Ferrante²†‡

Although JNK is a potential target for treating chronic inflammatory diseases, its role in T lymphocyte function remains controversial. To overcome some of the previous limitations in addressing this issue we have used the recently described transactivator of transcription-JNK-interacting protein (TAT-JIP) peptide, a specific inhibitor that was derived from the minimal JNK-binding region of the scaffold protein, JNK-interacting protein 1 (JIP-1), coupled to the short cell-permeable HIV TAT sequence. Pretreatment of purified human T lymphocytes with the TAT-JIP peptide inhibited the phosphorylation of endogenous Jun activated by PHA-PMA. This was associated with a corresponding inhibition of lymphoproliferation, and of IL-2, IFN-γ, lymphotoxin, and IL-10 cytokine production. Similar results were also found using mouse splenic T cells. Examination of the specificity of TAT-JIP revealed that although the peptide was more selective than the pharmacological inhibitor, SP600125, it also inhibited cyclin-dependent kinase 2, p70 ribosomal protein S6 kinase, and serum and glucocorticoid-regulated kinase activity. Nevertheless, these results demonstrate for the first time the ability of the TAT-JIP peptide to inhibit the JNK pathway and the phosphorylation of Jun in intact cells, thereby preventing the activation of the transcription factor, AP-1, and the production of Th1 and Th2 cytokines. Thus JNK potentially could be a target for the development of drugs for the treatment of autoimmune inflammatory diseases. The Journal of Immunology, 2008, 181: 7300–7306.

The JNK signaling pathway has been implicated in the pathogenesis of chronic inflammatory diseases, such as rheumatoid arthritis and atherosclerosis (1–4). JNK is activated following phosphorylation of Thr-183 and Tyr-185 by MAPK kinase (MKK)⁴ and MKK7 (5) and is responsible for the phosphorylation of serine 63 and serine 73 in the transactivation domain of c-Jun (6, 7).

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C-Jun N-terminal kinases, a subfamily of the MAPK, are activated by external stimuli including growth factors, inflammatory cytokines, and cellular stress (1–4). JNK is activated following phosphorylation of Thr-183 and Tyr-185 by MAPK kinase (MKK)⁴ and responsible for the phosphorylation of serine 63 and serine 73 in the transactivation domain of c-Jun (6, 7).

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35 min. PBMC were then harvested from the top band. T cells were purified by a combination of density gradient separation and adhesion to nylon wool columns as described previously (27). T cell purity was >98% as determined by FACScan analysis, and viability was >99% as determined by trypan blue dye exclusion. Splenic T cells were prepared from BALB/c mice essentially as described previously (28).

**Lymphoproliferation**

Fifty microliters of purified T cells (2 x 10^5 cells, 4 x 10^6/ml) were incubated with 50 l of TAT-JIP peptide (1–50 μM) or SP600125 (1–50 μM) for 30 min in 96-well U-bottom plates (Linbro; Flow Laboratories; Ref. 29). T cells were then stimulated for 48 h with PHA (2 μg/ml; Murex Diagnostics and PMA (10 ng/ml; Sigma-Aldrich)) diluted in RPMI 1640 containing 5% heat-inactivated blood group AB serum (5% AB serum) at 37°C in 5% CO2 and high humidity. Six hours before harvesting, 1/2 Ci [methyl-3H]thymidine (25 Ci/mmol; Amersham Life Sciences) diluted in RPMI 1640 (5% AB serum) was added to the cells. Supernatants were removed for cytokine determination. Cells were then harvested and incorporated radioactivity was measured using a Wallac liquid scintillation beta counter (Wallac 1409).

**FIGURE 1.** A, Active JNK in purified human peripheral blood T lymphocytes stimulated with PHA-PMA over 60 min. T lymphocytes were stimulated with PHA-PMA at 37°C/5% CO2 over a 60-min period. Samples were examined for phosphorylated JNK by Western blotting using anti-phospho-JNK (G-7) Ab. Blots were reprobed with β-actin Ab to establish equal loading. Data are presented as mean ± SEM of three experiments. ***, p < 0.001, Dunnnett’s Multiple Comparison Test.** B, Phosphorylation of jun in purified human peripheral blood T lymphocytes stimulated with PHA-PMA over 24 h. T lymphocytes were stimulated with PHA-PMA over a 24-h period. Samples were examined for phosphorylated jun by Western blotting using anti-phospho-c-jun Ab. Blots were reprobed with β-actin Ab to establish protein loading. Data are presented as mean ± SEM of three experiments. **, p < 0.05, **, p < 0.01, Dunnnett’s Multiple Comparison Test.

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<th>T Cell Function</th>
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<th>JNK 1 Mouse (Ref. 13)</th>
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<th>JNK1/JNK2 Mouse (Ref. 16)</th>
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*Studies were conducted with splenic mouse or human peripheral blood T cells. Arrows indicate a requirement (up-regulation or down-regulation) of JNK1 or JNK2 isoforms and the dash (−) signifies no role in T cell lymphoproliferation and cytokine production. Blank spaces indicate that the corresponding cytokine was not measured. The role of JNK1 and JNK2 are results from published data using JNK1<sup>-/-</sup> and JNK2<sup>-/-</sup> mice (Refs. 12–16). Results from human T cells are from the present study.
Cytokine determination

IFN-γ, IL-2, lymphotoxin (LT), and IL-10 levels were determined using cytometric bead array (BD Cytometric Bead Array Flex Sets; BD Biosciences) according to the manufacturer’s instructions. In brief, 50 μl of capture bead suspension and 50 μl of PE detection reagent were added to an equal amount of sample or standard dilution and incubated for 2 h at room temperature in the dark. Subsequently, samples were washed and centrifuged at 200 × g at room temperature for 5 min. Supernatant was discarded and 150 μl of wash buffer was added. Samples were analyzed on a BD FACSArray Bioanalyzer (BD Biosciences).

Western blotting for phosphorylated JNK/jun

Samples were examined for phosphorylated JNK and phosphorylated jun as described previously (29). In brief, T cells (1 × 10^6, 1 × 10^7/ml) were pretreated with TAT-JIP or SP600125 for 30 min before stimulation with PHA-PMA at 37°C/5% CO₂ for 4 h. Samples were then examined for phosphorylated jun by Western blotting using anti-phospho-c-jun Ab. Blots were reprobed with β-actin Ab to establish protein loading. Data are presented as mean ± SEM of three experiments, *p < 0.05, **p < 0.01, Dunnett’s Multiple Comparison Test.

Measurement of cyclin-dependent kinase 2 (CDK2)/cyclin A, casein kinase 1 (CK1), p70 ribosomal protein S6 kinase (p70S6K), ribosomal protein S6 kinase 1 (Rsk1), serine and threonine-protein kinase A (PKA), and dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) activity

Kinase profiler assays were performed by Millipore. In brief, each kinase (5–10 nM) was incubated with 8 mM MOPS (pH 7.0), 0.2 mM EDTA, and the respective substrate in a final reaction volume of 25 μl. CDK2/cyclin A was incubated with 0.1 mg/ml histone H1, whereas CK1, p70S6K, Rsk1, SGK, and DYRK were incubated with kinase-specific peptide KRRRA LSI(p)VASLPGL (200 μM), KKNRRTLTV (100 μM), KKKNRTLSSVA (30 μM), GRPRRTSSFAEGKK (30 μM), or casein (2 mg/ml), respectively. The reaction was initiated by the addition of 10 mM magnesium acetate and γ[32P]ATP, and after 40 min of incubation at room temperature it was stopped by the addition of 5 μl of 3% phosphoric acid solution. The reaction (10 μl) was then spotted onto a P30 filter mat and washed (3 times for 5 min) in 75 mM phosphoric acid and in methanol before drying and scintillation counting.

Statistical analyses

Experiments were conducted using cells from at least three different donors or mice. Statistical significance was evaluated using Bonferroni’s or Dunnett’s Multiple Comparison Test. A value of p < 0.05 was considered significant.
Results

Activation of the JNK pathway in human T lymphocytes

Initial studies examined the activation of JNK in human T cells stimulated with PHA-PMA. T cells were examined over a 60-min period for JNK activation by Western blotting using an Ab directed against phosphorylated forms of JNK. The results showed an initial small increase in JNK activation in the first 20 min, followed by an exponential increase in activity between 20 and 30 min (Fig. 1A). A plateau occurred over the next 15 min and JNK activity declined thereafter. This was the case for both the p46 and p54 isoforms of JNK.

JNK is predominantly responsible for the phosphorylation of jun (6, 7). Thus, to provide further evidence for the activation of the JNK pathway in human T lymphocytes, cells were stimulated with PHA-PMA over a 24-h period, and the samples were examined for jun phosphorylation by Western blotting using an Ab directed against phosphorylated forms of jun including c-jun, junB, and junD. As expected jun phosphorylation lagged behind JNK activation, with a prominent increase observed after 1 h of stimulation. This level of phosphorylated jun remained high until after 8 h of stimulation, and a reduction was detected after 24 h (Fig. 1B).

Effect of the TAT-JIP peptide on the phosphorylation of endogenous jun

The TAT-JIP peptide has previously been shown to inhibit the ability of JNK to phosphorylate jun in cell-free systems (19). However, we wanted to establish that the peptide inhibited the phosphorylation of endogenous jun in intact cells, more specifically in

FIGURE 4. Inhibition of T lymphocyte cytokine production by TAT-JIP. Purified human T lymphocytes were pretreated with TAT-JIP (10 μM) for 30 min and stimulated with PHA-PMA for 48 h at 37°C/5% CO2. Supernatants were harvested and cytokine levels were determined by cytometric bead array. Data are presented as mean ± SEM of three experiments. *, p < 0.05, **, p < 0.01, Dunnett’s Multiple Comparison Test. Cytokine production by cells stimulated with PHA-PMA was as follows: IFN-γ, 12726 ± 5181 pg/ml; IL-2, 42277 ± 14311 pg/ml; LT, 1279 ± 376 pg/ml; and IL-10, 414 ± 296 pg/ml.

FIGURE 5. Inhibition of T lymphocyte proliferation by TAT-JIP in mouse splenic T cells. Nylon wool-purified splenic T cells were pretreated with TAT-JIP (5, 10, 15, 20, 30, 50 μM) for 30 min and stimulated with PHA-PMA at 37°C/5% CO2 for 48 h. Cells were pulsed with 1 μCi of [methyl-3H]thymidine 6 h before harvesting, and incorporated radioactivity was measured. Data are presented as mean ± SEM of three experiments per performed in triplicate. *, p < 0.05, ***, p < 0.001, Bonferroni’s Multiple Comparison Test.

FIGURE 6. A, Comparison between the effect of TAT-JIP and SP600125 on PHA-PMA-induced human T cell proliferation. Purified human T lymphocytes were pretreated with TAT-JIP or SP600125 (1, 5, 10, 20 μM) for 30 min and stimulated with PHA-PMA at 37°C/5% CO2 for 48 h. Cells were pulsed with 1 μCi of [methyl-3H]thymidine 6 h before harvesting, and incorporated radioactivity was measured. Data are presented as mean ± SEM of three experiments. B, c-jun phosphorylation in purified human peripheral blood T lymphocytes pretreated with SP600125 and stimulated with PHA-PMA. T lymphocytes were pretreated with SP600125 before stimulation with PHA-PMA at 37°C/5% CO2 for 4 h. Samples were then examined for phosphorylated c-jun by Western blotting using anti-phospho-c-jun Ab. Blots were reprobed with β-actin Ab to establish protein loading. Data are presented as mean ± SEM of three experiments.
human T lymphocytes in response to PHA-PMA. This was investigated by pretreating the cells with the peptide (1–20 μM) before stimulation with mitogens. After 4 h of PHA-PMA stimulation, samples were examined for \( \text{jun} \) phosphorylation by Western blotting (Fig. 2). The TAT-JIP peptide inhibited the phosphorylation of endogenous \( \text{jun} \) in a concentration-dependent manner, with statistically significant inhibition observed between 10 and 20 μM.

**Effect on T lymphocyte function**

After establishing that the TAT-JIP peptide inhibited the ability of JNK to phosphorylate \( \text{jun} \), we investigated its effect on T lymphocyte proliferation. Cells were treated with peptide (1–20 μM) for 30 min before stimulation with PHA-PMA. Lymphoproliferation was measured after 48 h of culture. The data presented in Fig. 3 demonstrate that TAT-JIP-induced inhibition of T lymphocyte proliferation was evident at 10 μM and markedly increased at 20 μM, similar to the effect of the peptide on the phosphorylation of \( \text{jun} \). In contrast, the control peptide (50 μM), containing alanine substitution of two critical residues in the minimal JIP sequence (30), did not inhibit T lymphocyte proliferation (Fig. 3).

To gain a better understanding of the role of JNK in T cell function, we also examined the effect of the JNK inhibitor on cytokine production. Cells were treated with the TAT-JIP peptide (data shown for 10 μM only) for 30 min before stimulation with PHA-PMA. The peptide inhibited the production of LT, IFN-\( \gamma \), IL-2, and IL-10 production by 42, 46, 57, and 83%, respectively (Fig. 4).

Because the results we obtained were different from those using JNK1 and JNK2 knockout mice (12, 15, 16), we examined whether this was due to a variation in species. Mouse splenic T cells were prepared and treated with TAT-JIP (1–50 μM) for 30 min before stimulation with PHA-PMA. The results demonstrated that like human T cells, mouse splenic T cell proliferation was also inhibited by the TAT-JIP peptide in a concentration-dependent manner (Fig. 5).

The effects of TAT-JIP were compared with those of the pharmacological inhibitor of the JNK pathway, SP600125. There has been at present only one study that has reported the effects of this inhibitor on T lymphocytes (10). In our studies human peripheral blood T cells were pretreated with a concentration range of the inhibitor and then tested for proliferation in response to PHA-PMA. In contrast to the TAT-JIP peptide, SP600125 had no effect on PHA-PMA-induced T cell proliferation (Fig. 6A). This lack of effect was supported by the finding that SP600125 did not inhibit \( \text{jun} \) phosphorylation under these conditions (Fig. 6B). In contrast, both TAT-JIP and SP600125 inhibited neutrophil-mediated bacterial killing and suppressed \( \text{jun} \) phosphorylation (M. Yeh, V. Mukaro, C. Merantos, C. S. Hii, and A. Ferrante, unpublished observations).
Specificity of TAT-JIP for the JNK signaling pathway

Although the TAT-JIP peptide has been claimed to be highly selective for the JNK signaling pathway, it was important to examine the effect of the peptide on the activity of other kinases, previously shown to be inhibited by SP600125, including CDK2, p70S6K, CK1, SGK, Rsk1, and DYRK. This involved the Kinase Profiler Service performed by Millipore. The TAT-JIP peptide inhibited CDK2/cyclin A (Fig. 7A) and p70S6K (Fig. 7B) activity by >80% and SGK activity by 60% (Fig. 7C); however, the peptide did not inhibit CK1 (Fig. 7D), DYRK (Fig. 7E), and Rsk1 activity (Fig. 7F).

Discussion

To date there has been limited use of the TAT-JIP peptide in the examination of the JNK pathway in cellular function and no studies have been reported for T cells. For the first time we demonstrate the effectiveness of the TAT-JIP peptide in its ability to inhibit the JNK pathway in intact cells, specifically human T lymphocytes. PHA-PMA induced the phosphorylation of JNK and its substrate jun in purified T lymphocytes. Significant inhibition of jun phosphorylation occurred at 10 μM TAT-JIP peptide treatment. Thus the TAT-JIP peptide is an effective inhibitor and a useful tool in the investigation of the JNK pathway.

Our data establish a role for JNK in human T lymphocyte function. Purified human T lymphocytes directly stimulated with PHA-PMA showed activation of the JNK pathway in association with lymphoproliferation and cytokine production, notably, cytokines produced by human T lymphocytes was also dependent on JNK and that IL-10 production was particularly sensitive to the TAT-JIP peptide. This demonstrates the dependency on the JNK signaling pathway for cytokine production by human T cells.

It is widely accepted that the polarization of Th cell differentiation is at least in part determined by cytokine production (32). We have demonstrated that the JNK pathway influences IFN-γ production, a cytokine that promotes Th1 development (33). Interestingly, previous studies have revealed impairment in Th1 differentiation in JNK2−/− mice, which was due primarily to a reduction in IFN-γ secretion at the early stages of differentiation in IL-12-stimulated CD4+ T lymphocytes (15). Furthermore, CD4+ T lymphocytes isolated from JNK1−/− mice preferentially differentiate into Th2 cells (12). Thus the JNK pathway may also be important in regulating the balance between Th1 and Th2 cell type development.

The pharmacological JNK inhibitor, SP600125, was claimed to selectively target JNK1, 2, and 3 (10). However, in recent years the specificity of the JNK inhibitor has been questioned (11). SP600125 was found to be nonspecific because 13 of the 28 protein kinases tested were inhibited to a similar or greater extent than JNK, particularly SGK, p70S6K, CDK2, CK1δ, and DYRK1A (11). Furthermore, binding assays revealed that in addition to JNK1, 2, and 3, SP600125 bound to 36 of the 119 protein kinases that were tested (34).

In comparison to SP600125, the JIP-1-derived peptides are regarded as highly specific JNK inhibitors. Initial studies demonstrated that the 20-aa form of the JIP-1-derived peptide (25 μM) blocked JNK1, 2, and 3 phosphorylation of jun in vitro (21). Similarly, a previous study also demonstrated that the 10-aa form of the JIP-1-derived peptide did not inhibit ERK or p38 activity in vitro kinase assays involving neonatal rat ventricular myocytes (18). Furthermore, later studies found that with the exception of JNK and the associated MKK4 and MKK7, the 20-aa form of the JIP-1-derived peptide did not inhibit ERK, p38, protein kinase C, p34, calcium/calmodulin-dependent protein kinase, or protein kinase A at a concentration of 500 μM (23). Although we demonstrated that TAT-JIP is more selective than SP600125, for the first time this study establishes the limitations of this peptide.

The JIP peptide JNK inhibitors have been used to establish the role of JNK in a range of cellular functions and disease processes. These have been shown to block cerebral ischemia (23), death of pancreatic β cells (21), and acute trauma (25, 35), improve insulin resistance/glucose tolerance in diabetic mice (22) and prevent islet apoptosis following transplantation (36). In view of our findings on the ability of TAT-JIP to inhibit other kinases in addition to JNK, a reappraisal of the role of the JNK pathway is required. Although the finding that TAT-JIP inhibits CDK2 may be a reason for the inhibition of T cell proliferation, the evidence presented suggests that the JNK signaling pathway is the upstream event affected. Proliferation is dependent on IL-2, and it is known that IL-2 production requires the transcriptional factor AP-1 that contains jun (37).

It is evident that discrepancies exist in the role of JNK in T lymphocyte activation. We have now used another approach to examine its role in relation to human cells in which the JNK pathway was inhibited. This reaffirms the conclusion of Han et al. (9) that protection against joint damage in rheumatoid arthritis is likely to require the inhibition of both JNK1 and JNK2.

Our work has also raised further issues regarding SP600125, as it was found not to inhibit the PHA-PMA-induced proliferation of T cells and was incapable of inhibiting jun phosphorylation in human peripheral blood T cells. The only other study to address this question used the T cell line Jurkat (10). Only limited data were presented on the Western blots, which is not conclusive for
this inhibition because total jun protein was not examined. Furthermore, studies on cytokine production conducted on normal peripheral blood CD4+ T cells differentiated toward Th1 or Th2 types were restricted to one experiment and lymphoproliferation was not examined.

Current therapy for autoimmune diseases involves depletion of T cells and/or suppression of their responses. Pharmaceuticals such as anti-CD3 mAbs are used to deplete T lymphocytes, whereas others such as cyclosporin A and FK506 inhibit T lymphocyte activation, proliferation, and cell function by targeting intracellular signaling pathways (18, 38–41). The present findings suggest that JNK may be a useful target in the development of drugs for the treatment of autoimmune diseases. Of interest is the fact that the TAT-JIP peptide has already been tested as a potential therapeutic agent for diabetes, cerebral ischemia, and acoustic trauma-induced auditory hair cell death.

Disclosures

The authors have no financial conflict of interest.

References


