A Diketopiperazine Fragment of Human Serum Albumin Modulates T-Lymphocyte Cytokine Production Through Rap1

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Background: **Aspartyl-alanyl-diketopiperazine (DA-DKP) is generated by cleavage and cyclization from the N-terminus of human albumin during the preparation of commercial serum albumin product. Antigen-stimulated human T lymphocytes produce significantly lower quantities of interferon-γ and tumor necrosis factor-α after stimulation in vitro in the presence of DA-DKP.**

Methods: **T lymphocytes activated in the presence of DA-DKP were analyzed by pull-down western blot assay for the activation of the guanosine triphosphatase Rap1 and by quantitative immunoassay for the phosphorylated transcription factors ATF-2 (activating transcription factor-2) and c-jun, which regulate the production of interferon-γ and tumor necrosis factor-α.**

Results: **Exposure of human T lymphocytes to DA-DKP resulted in increased levels of active Rap1 and decreased activation factors relevant to the T-cell receptor signal transduction pathway and subsequently, decreased phosphorylated ATF-2 and c-jun expression.**

Conclusion: **The cyclized N-terminal fragment of human serum albumin, DA-DKP, can modulate the inflammatory immune response through a molecular pathway implicated in T-lymphocyte anergy.**

Key Words: **Anergy, Cytokine, Diketopiperazine, Rap1, T-lymphocyte.**

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Human serum albumin is used clinically to restore oncotic pressure in the critically ill. Its efficacy has been recently under considerable scrutiny with favorable outcomes in some studies contrasting with poor or no benefit in other reports.[1,2] Commercial preparations of human serum albumin (HSA) are heterogeneous in nature, including several molecular modifications arising from the collection, storage, and heat inactivation during the manufacturing process.[3] One of the modifications resulting from heat inactivation is the cleavage and cyclization of the N-terminal aspartate-alanine amino acid residues into aspartyl-alanyl-diketopiperazine (DA-DKP). We have previously demonstrated the ability of the DA-DKP derivative of HSA to decrease tumor necrosis factor (TNF)-α and interferon (IFN)-γ production from antigen-activated human T lymphocytes in vitro.[4] Here, we present a potential molecular basis for the anti-inflammatory activity of DA-DKP, which involves signal transduction proteins implicated in the induction of T-cell anergy.

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MATERIALS AND METHODS

Human T-Lymphocyte Cell Line (TRiPS) and In Vitro Culture

A cloned TRiPS, which produces cytokine in response to influenza hemagglutinin plus human leukocyte antigen-DR, was utilized for this study. Stimulation of cells in culture was performed as follows: 2×10^5 viable cells were precultured in the presence of DA-DKP in vitro in 1.0 mL Iscoves modified Dulbeccos minimal essential medium (IMDM) (American Type Culture Collection, Rockville, MD) for 1 hour at 37°C. Anti-CD3/anti-CD28 Dynabeads (Invitrogen, Carlsbad, CA) were then added at a predetermined concentration (2:1 bead to cell ratio to give 75%–80% optimal cytokine response) to activate T lymphocytes. For antigen stimulation, 25 μg/mL influenza A hemagglutinin peptide 307-319 plus 2×10^5 0.1% glutaraldehyde-fixed Epstein-Barr virus-transformed human leukocyte antigen-DR autologous peripheral blood leukocytes as antigen presenting cells in serum-free IMDM were added to effect near-optimal cellular activation (75%–80% maximal) as evidenced by cytokine production in prior studies. All cultures were incubated overnight at 37°C and an aliquot of the supernatant was then harvested for quantification by enzyme immunoassay (EIA).

NN Scientific, Ltd, St. Mellons, Cardiff, UK synthesized as Batch No. NNS 610 on October 15, 2006, the DA-DKP used in these experiments, which we analyzed to be >95% pure by anion exchange liquid chromatography negative electrospray mass spectroscopy.

Cytokine Assays

EIA for IFN-γ (BioSource, Camarillo, CA), interleukin (IL)-8, and TNF-α (Pierce-Endogen, Rockford, IL) were performed according to the manufacturer's instructions. Briefly, cell-free supernatants from overnight (16–18 hours) in vitro cultures were harvested and quantitated for specific cytokine release after stimulation via the T-cell receptor complex as described above. The sensitivity of the assays detecting IFN-γ averaged 10 pg/mL, for IL-8 was 10 pg/mL, and for TNF-α was 4 pg/mL.

Antibody Array for Phosphorylated Activation Factors

Custom antibody arrays for the detection of proteins relevant to T-lymphocyte activation were obtained from Hypromatrix (Worcester, MA) and used according to their instructions. Preliminary experiments indicated that 15 minutes in vitro stimulation of lymphocyte cell populations with either CD3/CD28 beads was optimal for induction of target protein phosphorylation. Briefly, 4 to 5 × 10^6 cells were preincubated in either DA-DKP or culture medium for 1 hour at 37°C, activated using anti-CD3/CD28 beads for 15 minutes, centrifuged to remove culture medium, and immediately lysed in mammalian cell-lytic buffer containing phosphatase inhibitors (Sigma, St. Louis, MO) and mammalian protease inhibitor cocktail (Amresco, Solon, OH). After a 4-hour incubation with cellular lysates at room temperature, arrays were washed with tris-buffered saline tween-20 and subsequently incubated for 2 hours with a rabbit pan-specific antibody recognizing serine-, threonine-, and tyrosine-phosphorylated proteins (Zymed, South San Francisco, CA). The arrays were again washed and incubated for 45 minutes with a anti-rabbit horse radish peroxidase (HRP)-conjugated antibody (Pierce-Endogen), washed extensively and reactivity detected by luminescence (ECL Plus, Amersham, Piscataway, NJ) and exposed using a Kodak 4000MM Image Station.

Expression of Active Rap1 by Western Blot Analysis

Active Rap1 was detected using the EZ-detect Rap1 activation kit (Pierce-Endogen) according to the manufacturer's instructions. Briefly, 4 to 5 × 10^6 viable cells were preincubated with or without DA-DKP for 1 hour in IMDM at 37°C, then stimulated with CD3/CD28 Dynabeads for 15 minutes, centrifuged to remove culture medium, and the cell pellets immediately dissolved in lysis buffer supplemented with protease inhibitors. Active Rap1 was selectively removed from the cellular lysates with GST-RalGDS-RBD fusion proteins, electrophoresed on 12% polyacrylamide gels, transferred to nitrocellulose, and detected using specific antibody and secondary HRP-conjugated reagents as described above. Active Rap1-specific luminescence was quantitated using a Kodak 4000MM Image Station (Kodak, Rochester, NY).

Quantitative Assay for Activated Transcription Factors

EIA kits were used according to the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, 2 × 10^6 viable cells were preincubated with or without DA-DKP in IMDM at 37°C, then stimulated for 30 minutes with CD3/CD28 Dynabeads, centrifuged to remove culture medium, and the cell pellets immediately dissolved in lysis buffer supplemented with protease inhibitors. Lysates were applied to microplate wells containing immobilized consensus DNA sequences specifically binding activated ATF-2 or c-jun and detected using antibodies specific for those respective proteins in a quantitative enzyme-linked assay.

Statistical Analysis

EIAs were performed in triplicate determinations and results shown as mean with SE. *p* values were calculated in Microsoft Excel (Microsoft Corp, Redmond, WA) using the student's *t* test. Correlation coefficients were calculated using linear regression in Excel. The significance level was set at 0.05.

RESULTS

DA-DKP Modulates Proinflammatory Cytokine Production in Human T Lymphocytes

We have previously shown that synthetic DA-DKP inhibited antigen-activated TNF-α production from a cloned TRiPS in a dose-dependent fashion.[4] DA-DKP decreased the production of proinflammatory INF-γ and TNF-α but not IL-8, irrespective of whether T cells were stimulated physi-

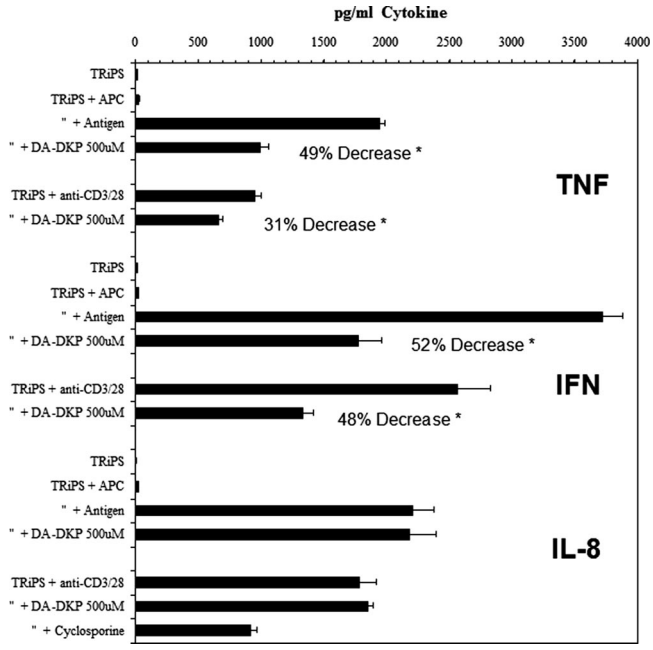

Fig. 1. *DA-DKP modulates proinflammatory cytokine production by human T lymphocytes. The cloned human T-lymphocyte cell line (TRiPS) was stimulated in vitro using either specific antigen plus autologous antigen presenting cells (APC) or anti-CD3/CD28 beads ± the presence of 500 μmol/L DA-DKP. Shown are the results from a single representative experiment repeated three times with indicated SE (*p< 0.05).*

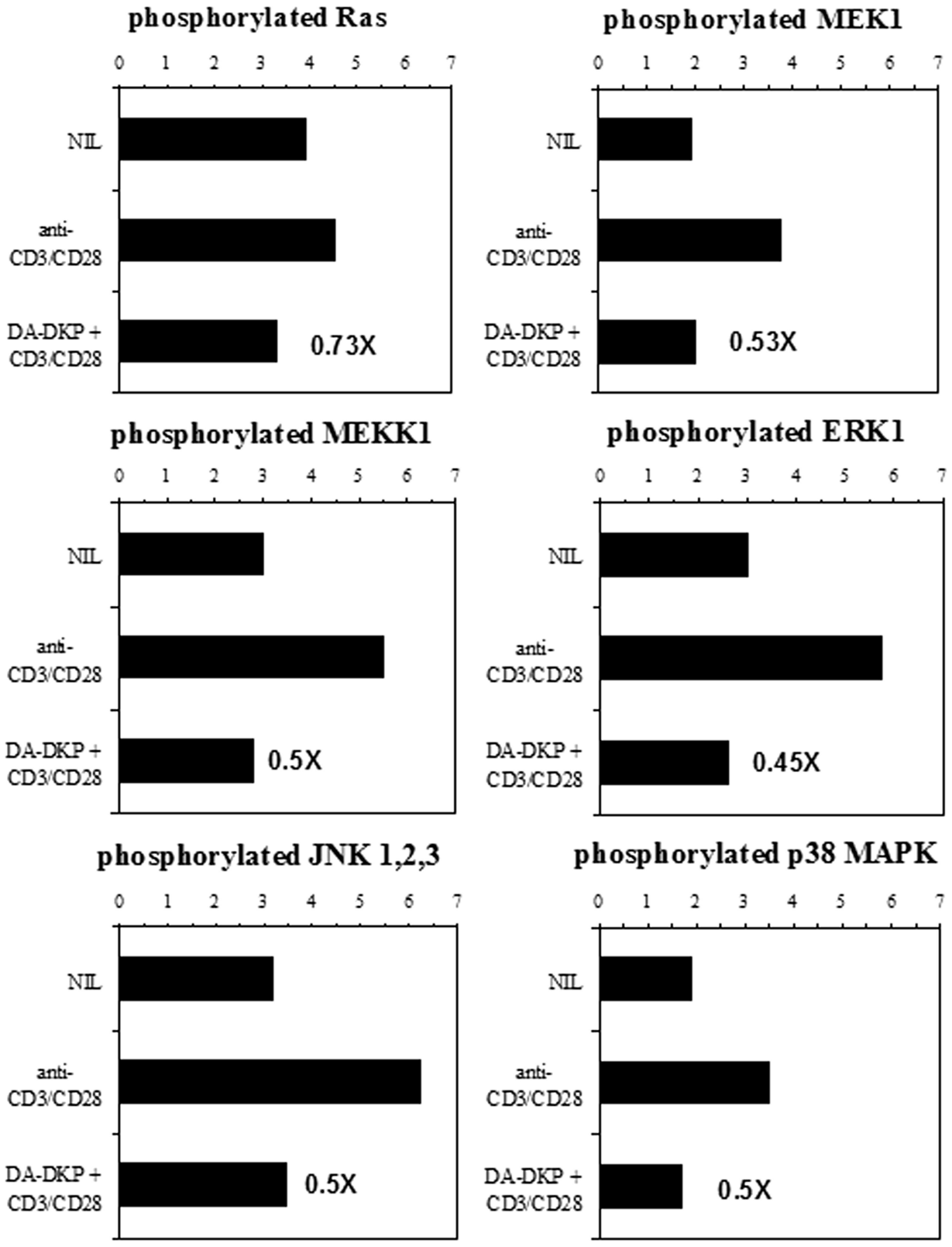

Fig. 2. *DA-DKP decreases the phosphorylation of activation factors expressed after stimulation through the T-cell receptor complex. The cloned human T-lymphocyte cell line (TRiPS) was stimulated in vitro using anti-CD3/CD28 beads ± the presence of 500 μmol/L DA-DKP. Shown are the results from a single representative experiment repeated twice with indicated fractional luminescence from cells cultured with DA-DKP plus anti-CD3/CD28 compared with cells stimulated with anti-CD3/CD28 alone.*

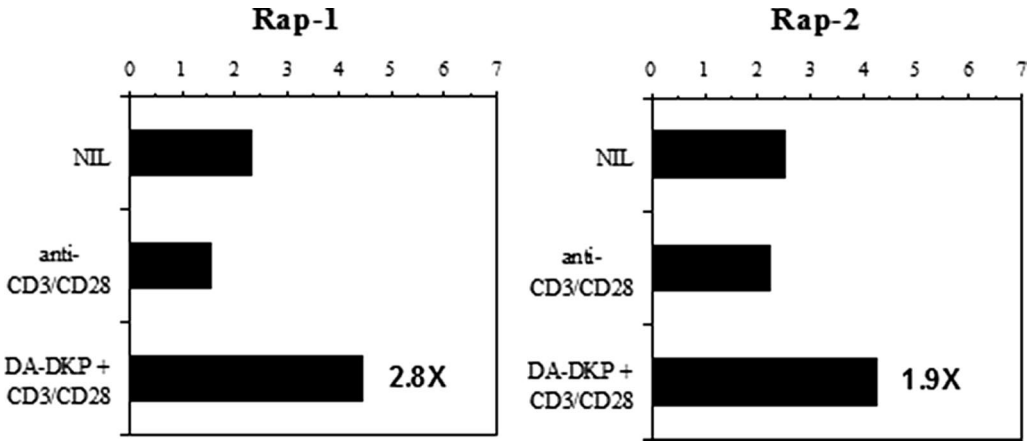

Fig. 3. *DA-DKP increases the phosphorylation of Rap1 and Rap2 after stimulation through the T-cell receptor complex. The cloned human T-lymphocyte cell line (TRiPS) was stimulated in vitro using anti-CD3/CD28 beads ± the presence of 500 μmol/L DA-DKP. Shown are the results from a single representative experiment repeated twice with indicated increase in luminescence from cells cultured with DA-DKP plus anti-CD3/CD28 compared with cells stimulated with anti-CD3/CD28 alone.*

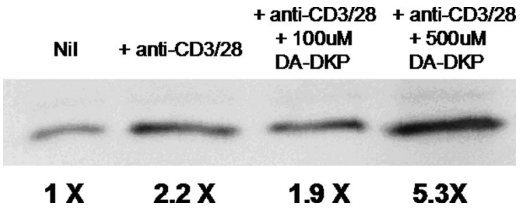

Fig. 4. *Active Rap1 is increased after stimulation of lymphocytes through the T-cell receptor complex in the presence of DA-DKP. The cloned human T-lymphocyte cell line (TRiPS) was stimulated in vitro using anti-CD3/CD28 beads ± the presence of 500 μmol/L DA-DKP. Shown are the results from a single representative pull-down western blot assay, repeated three times, for activated Rap1.*

ologically using specific antigen plus autologous antigen presenting cells or artificially using anti-CD3/CD28 beads (Fig. 1). A relatively high amount of DA-DKP (500 μmol/L) showed no significant effect on IL-8 release into culture supernatants but cyclosporine, a calcineurin inhibitor, did decrease IL-8 by approximately 50% from CD3/CD28-stimulated cells. IL-8 can be detected preformed in TRiPS cells by specific intracellular immunofluorescence (unpublished observations) and, as a consequence, cytokine release is not under transcriptional regulation. There was also no effect of 500 μmol/L DA-DKP on cell viability detected by Celltiter assay (Promega, Madison, WI, data not presented), which indicated that the decrease in INF-γ and TNF-α cytokine production was not the result of impaired cell viability. These results indicated that the modulation of T-lymphocyte cytokine production was subsequent to stimulation through the T-cell receptor complex and suggested that the inhibitory effect regulated the transcription of IFN-γ and TNF-α.

DA-DKP Effect on Phosphorylated Activation Factors

Preliminary experiments using anti-CD3/CD28 beads to activate the TRiPS cell line indicated that specific intracellular immunofluorescence of phosphorylated ERK 1/2 pT202/pY204 was detectable and maximal by 5 minutes poststimulation, with sustained expression for 30 minutes, and that of phosphorylated p38 mitogen-activated protein kinase (MAPK) pT180/pY182 was detectable by 15 minutes and maximal by 30 minutes poststimulation (data not presented). Therefore, we chose a 15-minute stimulation time with anti-CD3/CD28 beads for subsequent experiments to detect intracellular activation factors in this cell line. Protein array analysis revealed that preincubation with 500 μmol/L DA-DKP decreased the relative expression of phosphorylated ERK1, JNK1,2,3, MEK1, MEKK1, p38MAPK, and Ras after CD3/CD28-activation of cloned TRiPS cells to, or below, that observed in nil-stimulated cells (Fig. 2). To obtain sufficient amounts of cellular activation factors for array antibody capture (and western analysis) 4 to 5×10^6 cells were required. An increase in DA-DKP concentration to 500 μmol/L was necessary to observe an effect with the increased numbers of cells utilized; however, the ratio of DA-DKP to cells remained proportional to that used for the in vitro cultures for cytokine production (i.e., 50 μmol/L DA-DKP for 2×10^5 cells vs. 500 μmol/L DA-DKP for $4–5 \times 10^6$ cells).

Rap1 Activation

In contrast with the observed decrease in cellular activation factors, the G-coupled proteins Rap1 and Rap2 in CD3/CD28-activated TRiPS cells were found to be increased approximately twofold after DA-DKP preincubation (Fig. 3). However, phosphorylated Rap protein does not correlate with its guanosine triphosphatase (GTPase) activity, but GTP-bound Rap1 is active only when bound to its target protein Ral and can be accurately evaluated only by coprecipitation of the complex. Therefore, we used a Rap1/RalGDS pull-down assay to determine the effects of DA-DKP on the in vitro activation of TRiPS cells. Preculture in 100 μmol/L

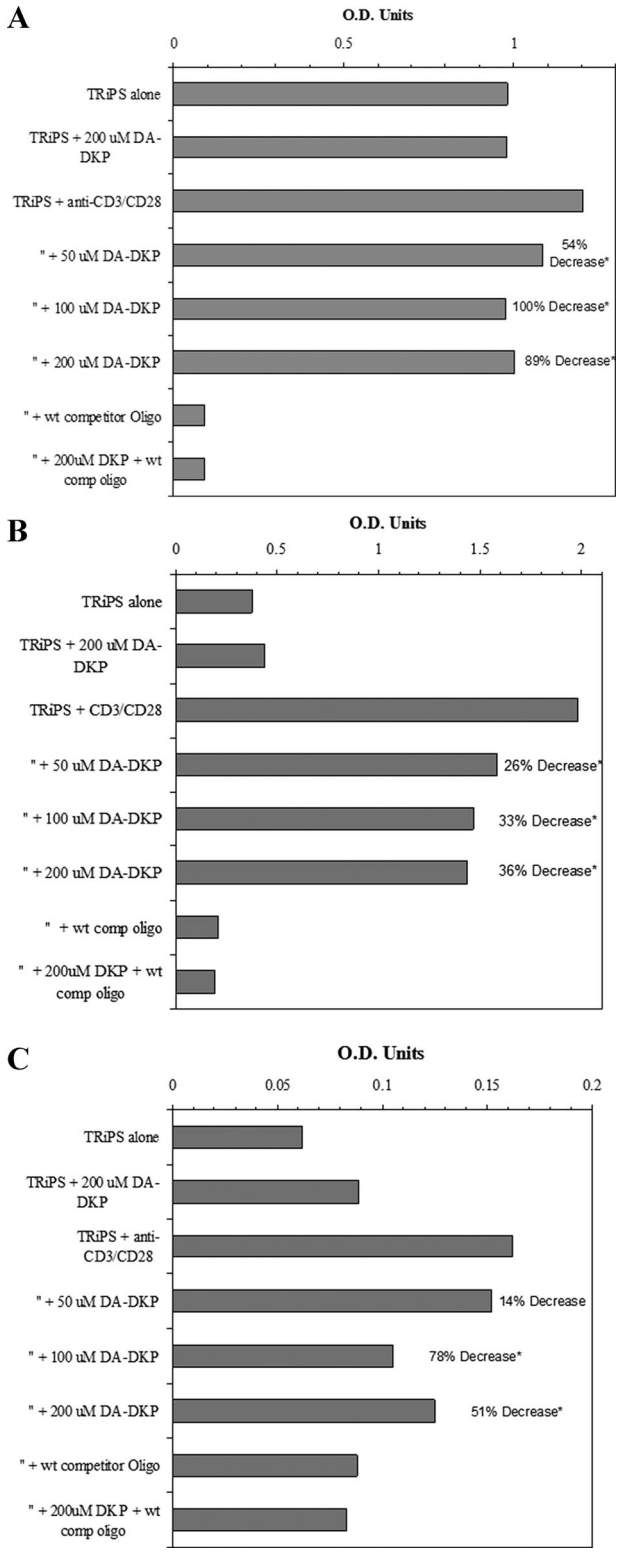

Fig. 5. *DA-DKP decreases the phosphorylation of transcription factors expressed after stimulation through the T-cell receptor complex. The cloned human T-lymphocyte cell line (TRiPS) was stimulated in vitro using anti-CD3/CD28 beads ± the presence of indicated amounts of DA-DKP. The indicated % decreased expression is relative to the increase in phosphorylated factor observed with anti-CD3/CD28 compared with nil stimulation plus DA-DKP. The wt comp (or, wild type competitor) oligo is supplied by the manufacturer and loss of signal indicates the specificity of the particular assay. The stimulation index (as the response seen with CD3/CD28 stimulation compared with nil stimulation) for ATF-2 was 1.2× (*A*), for c-jun was 5.3× (*B*), and for MEF-2 was 2.6× (*C*). Shown are the results from a single representative experiment repeated three times with the indicated significance (*$p < 0.05$).*

DA-DKP increased the expression of active Rap1 in three independent experiments an average of 2.2-fold and 500 μmol/L DA-DKP increased Rap1 an average of 3.9-fold compared with that using anti-CD3/CD28 alone. A representative experiment is shown in (Fig. 4).

Cytokine Transcription Factors

The decreased levels of phosphorylated cellular activation factors observed after CD3/CD28-mediated stimulation in the presence of DA-DKP suggested that the transcription of specific cytokines might be affected. The transcription factors ATF-2 (activating transcription factor-2) and c-jun are known to be activated by ERK 1/2, JNK, and p38 MAPK and regulate the transcriptional activation of both IFN-γ and TNF-α production in human T lymphocytes.[5,6] CD3/CD28-stimulated TRiPS cells precultured in vitro with DA-DKP expressed lower quantitative levels of the phosphorylated transcription factors ATF-2, c-jun, and MEF-2 (myocyte enhancer factor-2) (Fig. 5A–C, respectively). A varying degree of phosphorylated transcription factor expression was observed. ATF-2 levels were comparatively high in unstimulated cells and increased on average only 1.2 to 1.5× with anti-CD3/CD28 activation. During a series of three independent experiments, 50 μmol/L DA-DKP decreased the phosphorylation of ATF-2 induced by anti-CD3/CD28 an average of 35%, at 100 μmol/L effected a decrease of 63%, and 200 μmol/L DA-DKP an average of 83% (a representative experiment is shown in Fig. 5A). Phosphorylated c-jun levels were comparatively lower in nonstimulated cells and increased five to sevenfold with anti-CD3/CD28. DA-DKP at 50 μmol/L decreased the CD3/CD28-mediated increase in c-jun by an average of 21%, 100 μmol/L by 29%, and 200 μmol/L by an average of 46% (a representative experiment is shown in Fig. 5B). MEF-2 phosphorylation was observed at only 10% the levels of ATF-2 or c-jun, but DA-DKP also showed an inhibitory effect with 50 μmol/L effecting an average decrease of 12%, 100 μmol/L by 62%, and 200 μmol/L by 56% (a representative experiment is shown in Fig. 5C). In contrast, phosphorylated c-myc and Stat1 were detected at levels similar to MEF-2, being stimulated approximately twofold by CD3/CD28 activation of TRiPS cells, but were not affected by DA-DKP (data not presented).

DISCUSSION

Commercial HSA product is used clinically to restore hemodynamic stability in trauma and acutely ill patients. For

in vivo use, product is sterilized by heating in solution, which yields significant amounts of the diketopiperazine DA-DKP.[3] Synthetic DA-DKP by itself was found to decrease the in vitro production of the immunoinflammatory cytokines IFN-γ and TNF-α by activated human T lymphocytes.[4] Here, we show that DA-DKP modulates the production of proinflammatory cytokine in T lymphocytes through a mechanism involving the GTP-coupled protein Rap1 and the T-cell receptor signal transduction pathway. In concert with activation through the T-cell receptor complex, DA-DKP stimulated an increase in activated Rap1, decreased levels of the phosphorylated kinases MEK1, MEKK1, ERK1, JNK1,2,3, and p38MAPK, and decreased levels of the phosphorylated transcription factors ATF-2, c-jun, and MEF-2.

Stimulation of T lymphocytes through the T-cell receptor complex results in activation of the GTPase Ras, which in turn leads to phosphorylation-induced activation of Raf and MEK kinases[7] leading ultimately to cell division and cytokine production. Rap1 has been shown to play a critical role in the activation of T lymphocytes by regulating the amount of Raf available for interaction with Ras.[8] Presumably, either increased levels or a sustained expression of Rap1 after T-cell receptor ligation leads to anergy, a nonresponsive state in which the T-cell receptor-mediated signal transduction pathway through Ras is impaired.[9] Accordingly, stimulation of T lymphocytes through CD28, a cell surface costimulatory molecule (that is, in concert with the T-cell receptor), suppresses Rap1 activation[10] whereas CTLA-4 (cytotoxic T lymphocyte antigen-4), an inhibitory cell surface costimulatory ligand, activates Rap1.[11]

In the study presented here, DA-DKP-mediated Rap1 expression decreased phosphorylated ATF-2 and c-jun transcription factors, both of which are involved in the regulation of TNF-α gene transcription[5] and IFN-γ gene transcription.[6] In contrast, release of preformed IL-8 protein, which is not controlled by transcriptional regulation, was not affected by DA-DKP. Also, the IL-8 promoter is regulated by a different factor, RelA.[12] Apparently, DA-DKP induction of active Rap1 affects the phosphorylation of selected transcription factor pathways activated by T-cell receptor signaling and is not a global effect. We have in vitro cultured T lymphocytes in 500 μmol/L DA-DKP-supplemented culture media for up to 14 days with no loss of cell viability or function when removed from DA-DKP culture (unpublished observation).

Commercially prepared HSA has previously been shown to have in vivo and in vitro anti-inflammatory effects on nuclear transcription factors. In an animal shock model, 25% albumin resuscitation diminished neutrophil chemoattractant factor mRNA levels and NF-κB translocation after endotoxin challenge.[13] In vitro culture with HSA increased glutathione levels and decreased TNF-α-mediated NF-κB activation in human lung epithelial cells, fibroblasts, and monocyte-depleted PBMC (peripheral blood mononuclear cell).[14] We previously characterized the anti-inflammatory effect of HSA preparations on T-lymphocyte cytokine production and now present the molecular mechanism by which a single component found in significant amounts in commercial HSA can modulate the transcription of specific cytokine genes. The in vitro effect of DA-DKP was to activate the GTPase Rap1, which decreased the phosphorylation of activation factors involved in the T-cell receptor signal transduction pathway and resulted in decreased immunoinflammatory cytokine transcription. This effect was only found in concert with activation through the T-cell receptor, suggesting the effect in vivo would be to regulate the initiation of a T-lymphocyte immune response. Our studies to date have investigated only the in vitro effects of commercial albumin and DA-DKP on human peripheral blood mononuclear cells and TRiPS, and the in vivo consequences which HSA containing DA-DKP has on human patients remain to be evaluated. Clinically, administration of HSA has been demonstrated beneficial for acute lung injury[13,15–17] and sepsis,[1] conditions where TNF-α is considered to play a significant role.[18,19] The collective data suggest that the anti-inflammatory properties of commercial HSA products, which contain DA-DKP should be appropriately considered when applied to clinical use.

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