



Synergistic inhibition of T-cell activation by a cell-permeable ZAP-70 mutant and ctCTLA-4

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ABSTRACT

T-cell activation requires TcR-mediated and co-stimulatory signals. ZAP-70 participates in the initial step of TcR signal transduction, while a co-receptor, CTLA-4, inhibits T-cell activation. In previous studies, the overexpression of a ZAP-70 mutant (ZAP-70-Y319F) inhibited the TcR-induced activation of NFAT and IL-2 production, while Hph-1-ctCTLA-4 prevented allergic inflammation. To develop an effective immunosuppressive protein drug that blocks both TcR-mediated and co-stimulatory signaling pathways, a fusion protein of ZAP-70-Y319F and the Hph-1 protein transduction domain was generated. Hph-1-ZAP-70-Y319F inhibited the phosphorylation of ZAP-70-Tyr³¹⁹, LAT-Tyr¹⁹¹, and p44/42 MAPK induced by TcR stimulation, NFAT- and AP-1-mediated gene transcription, and the induction of CD69 expression and IL-2 secretion. Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4 synergistically inhibited signaling events during T-cell activation. This is the first report to demonstrate the synergistic inhibition of signals transmitted via TcR and its co-stimulatory receptor by cell-permeable forms of intracellular signal mediators.

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Introduction

T-cells mediate adaptive immune responses and thus play a crucial role not only in inducing normal immune responses against foreign antigens, but also in causing excessive immune responses responsible for autoimmune disorders, graft rejection, and allergy [1,2]. Therefore, the modulation of T-cells is one of the major approaches in the therapeutic treatment of various immune diseases.

The activation, differentiation, and effector functions of antigen-specific T-cells are induced by signals transmitted via the T-cell antigen and co-stimulatory receptors [3]. The overall orchestration of these events regulates the immune response. One of the most important features of initiation is the activation of T-cells. Upon activation of the T-cell receptor, Lck is recruited to the TcR-CD3 complex. This complex has cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) that are phosphorylated by Lck [4]. This leads to the rapid phosphorylation and activation of ZAP-70 on its several tyrosine residues [5,6].

These events have been implicated in both positive and negative regulation of ZAP-70 activity, which are coupled to downstream signaling pathways [7]. In particular, the phosphorylation

of Tyr³¹⁹ has been reported to be responsible for the upregulation of ZAP-70 function [8,9]. The overexpression of its mutant (ZAP-70-Y319F) in Jurkat T-cells and murine T-cell hybridoma cells showed significant impairment of the activation of nuclear-factor-activated T-cells (NFATs) and interleukin-2 (IL-2) [10,11].

CD28 is the major co-stimulatory molecule for the activation of T-cells. It is constitutively expressed on T-cells, while B7.1 and B7.2 are upregulated on the cell surface upon APC activation. CTLA-4 is a second receptor in T-cells for B7-1 and B7-2. It binds B7-1 with a 20-fold higher affinity as compared to CD28 and inhibits the T-cell response by competing with CD28 [12]. The cytoplasmic domain of CTLA-4, which contains a tyrosine motif, is 100% conserved among different species, suggesting that this domain is important for the negative regulation of T-cell activation by CTLA-4. It was demonstrated in previous studies that Hph-1-ctCTLA-4 constitutes an effective immunosuppressive protein drug for potential use in the treatment of allergic asthma [13].

CD3-IgG and CTLA-4 Ig are potent immunosuppressive agents that act by modulating the TcR complex [14]. Their properties have been exploited for the treatment of autoimmune diseases. In addition to their potent capacity to alter immune responses via TcR modulation, these antibodies also exhibit unwanted toxicity. Since the blockage of TcR signaling by mAb induces nonspecific inhibition of all signaling pathways that are downstream of TcR activation, it is necessary to develop a molecule that can inhibit certain signaling pathways with high specificity. Although many key intracellular mediators are specifically involved in signaling pathways in T-cell

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activation and could be optimal target molecules, these proteins cannot be delivered across the cell membrane *in vitro* and *in vivo*.

Hph-1 contains a domain classified as a protein transduction domain (PTD). This domain works as a protein vector, allowing the transduction of macromolecules across the otherwise impermeable, double-layered cell membrane [15–17]. It has been extensively studied and can transport large biologically active proteins such as β -galactosidase [18].

In this study, a cell-permeable form of the dominant negative tyrosine mutant of ZAP-70 (Hph-1-ZAP-70-Y319F) was developed, and its potential to inhibit TcR-mediated signaling pathways was examined. Hph-1-ZAP-70-Y319F effectively suppressed all of the signaling events associated with TcR stimulation in Jurkat T-cells as well as in human CD4⁺ and CD8⁺ T-cells. To block both TcR and co-stimulatory signals, Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4 were used together on Jurkat T-cells and human CD4⁺ and CD8⁺ T-cells. Surprisingly, Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4 suppressed T-cell activation to almost background levels. These results demonstrate that the blockage of activation signals through both the TcR complex and the co-stimulatory receptor by Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4 is an effective and synergistic therapeutic approach for autoimmune diseases and transplant rejection.

Materials and methods

Construction of the *Escherichia coli* expression vector. The full-length ZAP-70 human gene was amplified from the Human T-Cell Library. PCR was performed using Pfu DNA polymerase (Stratagene). The PCR product was cloned into pGELysRS and confirmed via restriction analysis and DNA sequencing. The pGELysRS was a gift from Dr. Baik L. Seong (Yonsei University).

Purification of recombinant protein. For the purification of Hph-1-ZAP-70-Y319F in the HMS174 *E. coli* strain, the culture was grown at 25 °C with shaking, and isopropyl- β -thiogalactopyranoside (IPTG) (400 μ M) was added at an OD of 0.5 (A_{600}). At 24 h post-induction, the cells were harvested by centrifugation (6000 rpm for 10 min, at 4 °C), resuspended in lysis buffer (6-M GuHCl, 10-mM Tris-Cl, pH 8.0, 100-mM NaH₂PO₄), and sonicated. The lysate was clarified by centrifugation (13,000 rpm for 10 min, at 4 °C) and loaded on a HisTrap chelating column (Qiagen). The column was washed with a wash buffer (pH 6.3) and eluted with an elution buffer (pH 4.5). The eluted proteins were desalted using PD-10 Sephadex G-25 (Amersham Pharmacia). They were then supplemented with 10% glycerol, separated into aliquots, and flash-frozen at –80 °C [19].

Cell preparation. Primary CD4⁺ or CD8⁺ T-cells from human peripheral-blood mononuclear cells were purified with a MACS column, using CD4 or CD8 microbeads (Miltenyi Biotec). To generate stable transfectants, 40 μ g of NFAT and AP-1 SEAP plasmids were transfected into Jurkat T-cells via electroporation (250 V/960 μ F) [20]. Stable transfectants were independently selected four times in the presence of 2.2-mg/ml G418 (Invitrogen) for two weeks.

Cell viability assay. Cell survival was determined using a WST-8 assay kit. Briefly, cells (5×10^4 /well) in 96-well plates were incubated with RPMI-1640 and purified protein overnight. After the incubation, WST-8 reagents were added to the culture, and 450-nm absorbance values were obtained after 2 h of incubation. The absorbance measured in this assay correlates with the number of living cells [21].

Western blots. Cell lysates were prepared from cells treated with purified protein and were fractionated using SDS-PAGE. After electrophoresis, the proteins were electrotransferred to an Immobilon-P transfer membrane (Millipore) and blotted with anti-ZAP-70 mAb (Cell Signaling) and anti-GAPDH mAb (Cell Signaling), anti-

phospho-ZAP-70 (Tyr319) mAb (Cell Signaling), anti-phospho-LAT (Tyr191) mAb (Cell Signaling), anti-phospho-p44/42 MAPK (Cell Signaling), and β -Actin mAb (Cell Signaling).

T-cell activation. Tissue culture plates (12-well, flat-bottom) were precoated with 500 μ l of anti-CD3 (1 μ g/ml) for 3 h at 37 °C and were then washed twice with 1 ml of PBS. Jurkat T-cells (2×10^5 /microtube/100 μ l) treated with purified protein were incubated with anti-CD28 for 20 min on ice and were then incubated with immobilized anti-CD3 for 18 h in complete media. Human primary T-cells and transfectants were also activated as described above. Supernatants were then obtained and stored at –70 °C until the determination of their cytokine content.

SEAP assay. After activation, the activity of SEAP in the cell-free supernatant was evaluated using a chemiluminescent method with a SEAP detection kit (Clontech). In brief, 15 μ l of the culture media were mixed with 45 μ l of the 1 $\times$ dilution buffer and incubated for 30 min at 65 °C. After the incubation, the samples were mixed with 60 μ l of the assay buffer and left at room temperature for 5 min. Sixty microliters of a chemiluminescent enhancer containing 1.25-mM CSPD chemiluminescent substrate were then added. After incubating in the dark for 10 min, the samples were subjected to a SEAP activity assessment.

ELISA for IL-2 detection in the culture supernatant of T-cells. The IL-2 level in the culture supernatant was quantified using an ELISA kit (R&D Systems). The absorbance was read at 450 nm using an ELISA plate reader.

Flow cytometric analysis of CD69 expression. After 18 h of activation, the cells were stained for 20 min on ice with anti-CD69-PE antibody (Becton–Dickinson). After washing with PBS, the cells were analyzed using FACScalibur (Becton–Dickinson).

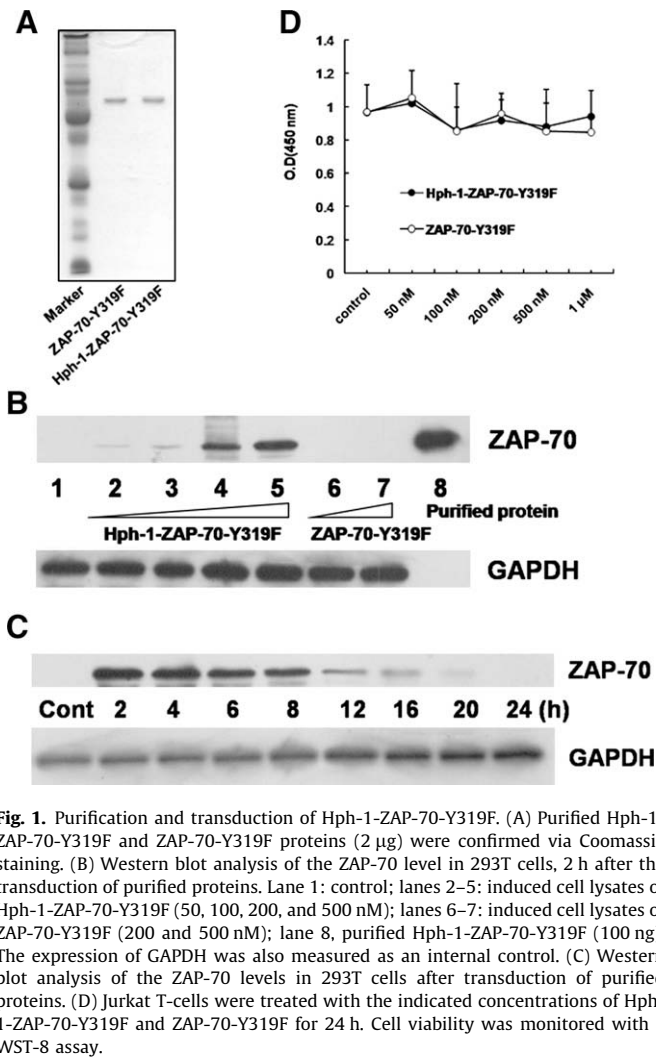
Results

Effective transduction of Hph-1-ZAP-70-Y319F into cells

Novel, Human-origin Hph-1-PTD was previously identified from the transcription factor, Hph-1. The PTD can facilitate transduction of the protein into the cytoplasm and nucleus of cells *in vitro* and *in vivo*, via systemic or various local administration routes [13]. To generate a cell-permeable form of the ZAP-70 mutant (Hph-1-ZAP-70-Y319F), which was demonstrated to inhibit the TcR-specific signaling pathway in a transfection study, Hph-1-ZAP-70-Y319F was expressed in an *E. coli* system and was purified under denaturing conditions (Fig. 1A). To examine the transduction efficiency of Hph-1-ZAP-70-Y319F in the cells, various concentrations of Hph-1-ZAP-70-Y319F were incubated with 293T cells for different time periods. Hph-1-ZAP-70-Y319F was transduced into the 293T cells in a concentration- and time-dependent manner, while ZAP-70-Y319F without Hph-1-PTD was not delivered into the cells (Fig. 1B and C). The transduced Hph-1-ZAP-70-Y319F protein easily reached its maximum concentration in the cells, which then started decreasing gradually 6 h post-transduction (Fig. 1C). To determine whether the transduced Hph-1-ZAP-70-Y319F had any adverse effect on cell viability, Jurkat T-cells were treated with various concentrations of Hph-1-ZAP-70-Y319F or ZAP-70-Y319F proteins for 24 h, and the level of cell death was analyzed using a WST-8 assay. No signs of increased cell death were detected using a significantly higher dose of Hph-1-ZAP-70-Y319F (Fig. 1D). These results suggest that the cell-permeable form of ZAP-70-Y319F can be efficiently transduced into cells without causing nonspecific cell death.

Suppression of TcR-mediated signals by Hph-1-ZAP-70-Y319F

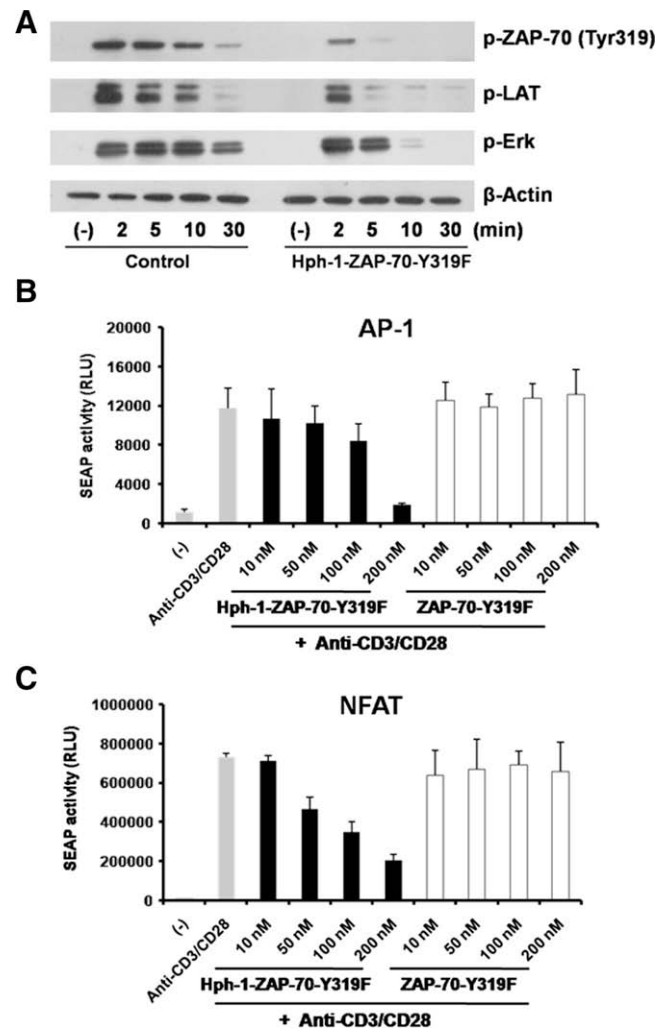
To examine whether the transduced Hph-1-ZAP-70-Y319F protein could inhibit tyrosine phosphorylation of key signal mediators



proximal to the TcR complex, Jurkat T-cells were treated with 500-nM Hph-1-ZAP-70-Y319F for 2 h and were then activated with anti-CD3 and anti-CD28 mAb, followed by immunoblotting for phosphotyrosine. Tyrosine phosphorylation of ZAP-70, LAT, and p44/42 (Erk1 and Erk2) was found to be greatly reduced by Hph-1-ZAP-70-Y319F (Fig. 2A).

AP-1 and NFAT play a key role in the regulation of cytokine and cell-surface receptor genes during immune responses and are activated after the cross-linking of the TcR in T-lymphocyte [22]. To examine whether Hph-1-ZAP-70-Y319F affected the transcription factors AP-1 and NFAT, AP-1-SEAP- and NFAT-SEAP-transfected Jurkat T-cell lines were generated. After pretreatment of Hph-1-ZAP-70-Y319F and ZAP-70-Y319F with AP-1 and NFAT transfectants, cells were activated with anti-CD3 and anti-CD28 mAb for 18 h, and each supernatant was harvested for a reporter gene assay. As a result, Hph-1-ZAP-70-Y319F suppressed AP-1 and NFAT-mediated gene transcription in a concentration-dependent manner. ZAP-70-Y319F showed no effect, however, on AP-1 and NFAT (Fig. 2B and C).

IL-2 is the most critical cytokine in the development of an adaptive immune response, and the expression of IL-2 is mainly induced by AP-1, NFAT, and NF- κ B [23]. The early activation marker CD69 is upregulated rapidly after TcR stimulation and disappears soon after, upon termination of the stimulation [24]. To investigate the inhibitory function of Hph-1-ZAP-70-Y319F on IL-2 cytokine production and on the induction of surface expression



of CD69, Jurkat T-cells and human CD4⁺ and CD8⁺ T-cells were stimulated with anti-CD3 and anti-CD28 mAb in the presence of Hph-1-ZAP-70-Y319F or ZAP-70-Y319F for 18 or 48 h, respectively. The supernatant was then analyzed for IL-2 using an ELISA. As shown in Fig. 3, Hph-1-ZAP-70-Y319F inhibited IL-2 production in a concentration-dependent manner, while ZAP-70-Y319F without Hph-1 PTD had no effect. Taken together, Hph-1-ZAP-70-Y319F strongly inhibited tyrosine phosphorylation of key intracellular signal mediators, activation of transcription factors, and IL-2 production. CD69 expression followed the pattern of IL-2 expression upon Hph-1-ZAP-70-Y319F induction (Supplementary data, Fig. S1).

Synergistic inhibition of signaling pathways for T-cell activation by Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4

TcR-mediated signaling is regulated by ZAP-70, and co-stimulatory signaling through CD28 is negatively regulated by CTLA-4. Therefore, blocking these two signaling pathways at the same

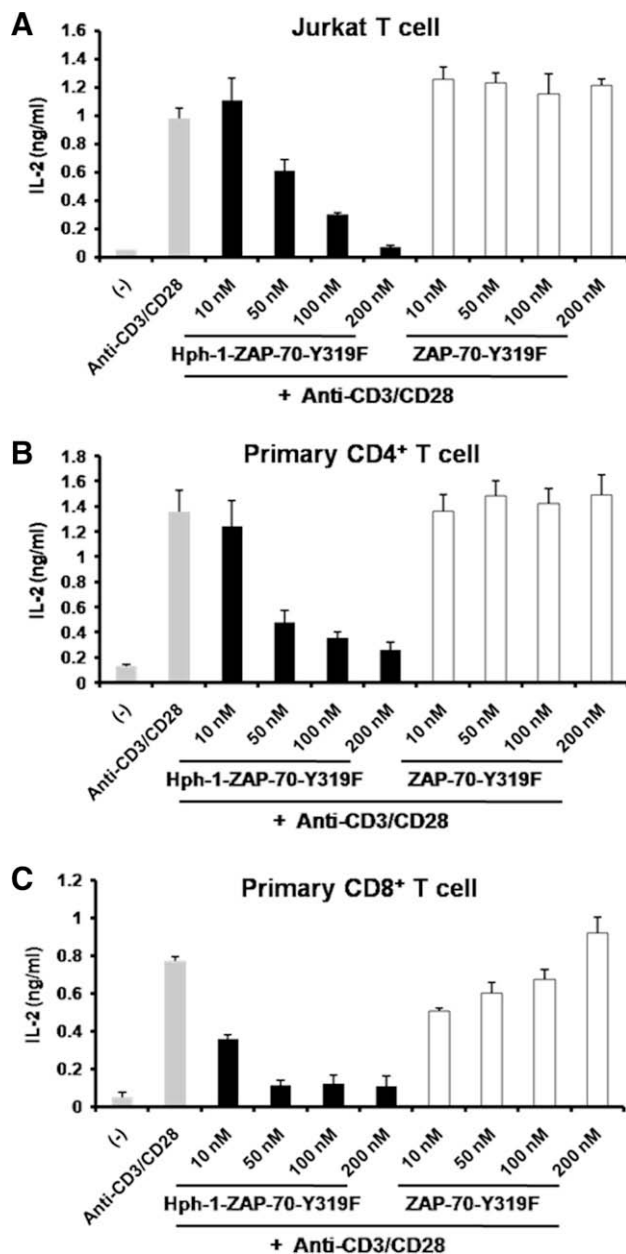


Fig. 3. Effect of Hph-1-ZAP-70-Y319F and ZAP-70-Y319F on IL-2 production. (A) Observation of IL-2 production by Jurkat T-cells incubated with Hph-1-ZAP-70-Y319F and ZAP-70-Y319F (10, 50, 100, and 200 nM) and activated with plate-bound anti-CD3 plus anti-CD28 for 18 h. Observation of IL-2 production by primary CD4⁺ T-cells (B) and primary CD8⁺ T-cells (C) incubated with purified proteins (10, 50, 100, and 200 nM) and activated with plate-bound anti-CD3 plus anti-CD28 for 48 h. The error bars indicate the standard error of the mean from three biological replicates.

time can completely inhibit T-cell activation. In previous studies, the cell-permeable form of the cytoplasmic domain of CTLA-4 (Hph-1-ctCTLA-4) caused a marked reduction in T-cell activation, and this inhibition was specific to co-stimulatory signaling [13,25]. These results suggest the possibility that Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4 may inhibit T-cell activation in a synergistic manner to block both TcR and co-stimulatory signaling. To test this possibility, Jurkat T-cells and human CD4⁺ and CD8⁺ T-cells were treated with Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4, followed by T-cell activation with anti-CD3 and anti-CD28 mAb. The induction of IL-2 secretion was then measured. As shown in Fig. 4, the level of IL-2 secretion was sig-

nificantly inhibited, measuring at only background levels, when the T-cells were co-treated with Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4. CD69 expression followed the expression pattern of IL-2 upon Hph-1-ZAP-70-Y319F induction (Supplementary data, Fig. S2). These results confirm that the blockage of both TcR-mediated and co-stimulatory signals by Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4 is much more effective in inhibiting T-cell activation than the blockage by either Hph-1-ZAP-70-Y319F or Hph-1-ctCTLA-4 alone.

Discussion

In this study, a first attempt to inhibit T-cell activation was made, using two cell-permeable forms of T-cell-specific intracellular signal mediators: a dominant negative mutant form of ZAP-70 and the cytoplasmic domain of CTLA-4. Hph-1-ZAP-70-Y319F was efficiently transduced into cells in a dose- and time-dependent manner and suppressed TcR-mediated activation signaling events by reducing the phosphorylation of ZAP-70-Tyr³¹⁹, LAT-Tyr¹⁹¹, and p44/42 (Erk1 and Erk2). It also inhibited the TcR-induced transcriptional activity of AP-1 and NFAT, the activation and induction of IL-2 secretion, and CD69 expression in Jurkat T-cells and human CD4⁺ and CD8⁺ T-cells. Most importantly, it was confirmed that co-treatment of these T-cells with Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4 resulted in more potent inhibition of CD69 expression and IL-2 production compared to treatment using either Hph-1-ZAP-70-Y319F or Hph-1-ctCTLA-4 alone.

Although chemical immunosuppressors such as CsA and FK506 are frequently used in transplantation and treatment of autoimmune diseases, their nonspecific inhibition of immune reactivity and inflammation results in a considerable number of side effects and does not lead to a permanent cure [26]. The autoimmune diseases and graft rejections targeted by these types of drugs are caused by T-cells, which play a major role in the adaptive immune response. Therefore, the ultimate purpose of current immunosuppressive drugs is to block T-cell activation in a specific manner. Since T-cell activation requires a combination of TcR-mediated and co-stimulatory signals, the blocking of TcR and co-stimulatory pathways has been an appealing strategy for inducing tolerance in autoimmune diseases and graft rejection. In fact, the blockage of CD3 signaling or B7/CD28 co-stimulation has proven very efficacious in preventing the induction of numerous animal models of human autoimmune disease and in organ transplantation [27,28]. Based on these studies, we suggest blocking both the CD3 and co-stimulatory signals in combination for more efficient immunosuppression.

In this study, two T-cell-specific intracellular signal mediators known to inhibit T-cell activation were chosen and generated in cell-permeable forms. While all of the signaling events involved in T-cell activation were substantially inhibited by either Hph-1-ZAP-70-Y319F or Hph-1-ctCTLA-4, the combination treatment of Jurkat T-cells and human CD4⁺ and CD8⁺ T-cells with both Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4 exerted synergistic inhibitory effects rather than triggering activation.

The data that were obtained in this study may suggest new regulatory potential for key signal mediators involved in T-cell activation, leading to the selective modulation of specific signaling pathways and the discovery of a novel immune modulator for the treatment of transplant patients and various autoimmune diseases. Moreover, the ability of cell-permeable forms of signal mediators to be delivered through local administration routes (e.g., airways, skin, eyes) provides a great advantage in terms of the development of novel immunosuppressive protein drugs with less toxicity to the other organs. The *in vivo* immunosuppressive activity of Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4 is being investi-

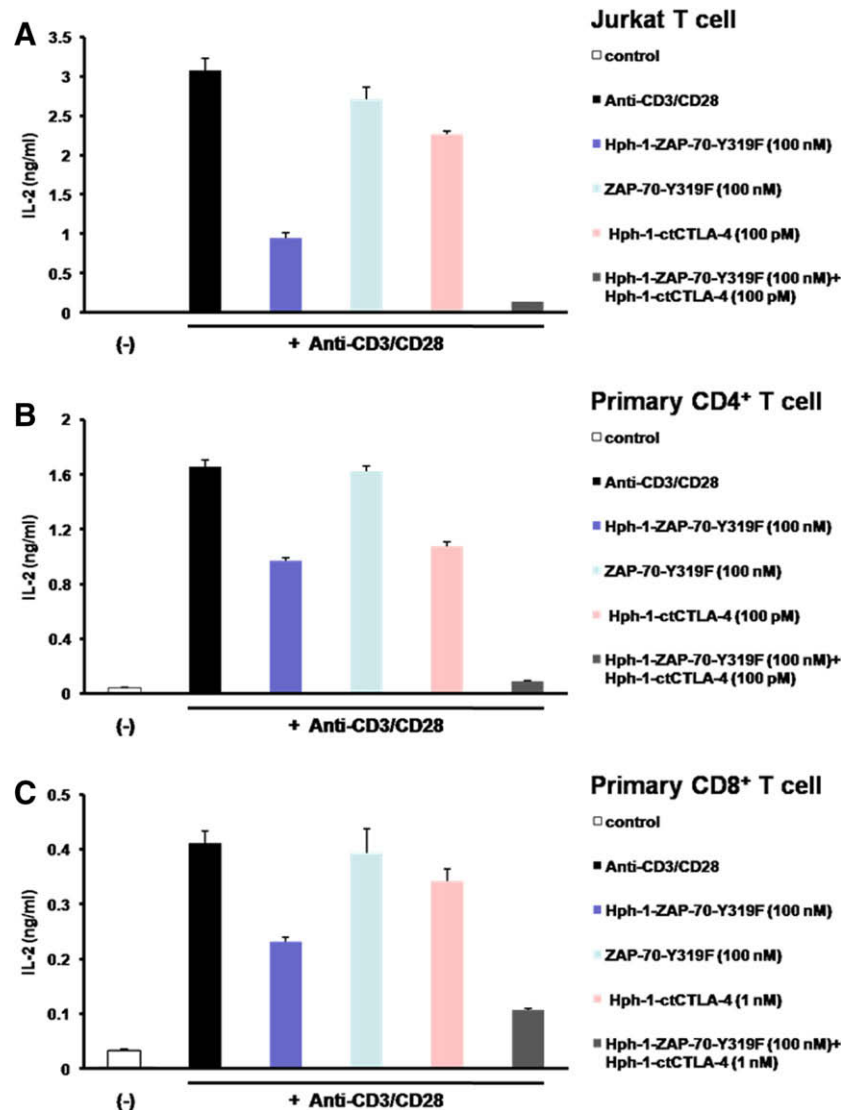


Fig. 4. Synergistic effects of Hph-1-ZAP-70-Y319F and Hph-1-ctCTLA-4. (A) Observation of IL-2 production by Jurkat T-cells incubated with Hph-1-ZAP-70-Y319F (100 nM) and Hph-1-ctCTLA-4 (100 pM) and activated with plate-bound anti-CD3 plus anti-CD28 for 18 h. Observation of IL-2 production by primary CD4⁺ T-cells (B) and primary CD8⁺ T-cells (C) incubated with purified proteins and activated with plate-bound anti-CD3 plus anti-CD28 for 48 h. The error bars indicate the standard error of the mean from three biological replicates.

gated in various disease models related to immune system dysfunction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2009.02.046](https://doi.org/10.1016/j.bbrc.2009.02.046).

References

- [1] C.J. Hackett, H.B. Dickler, Immunologic tolerance for immune system-mediated diseases, *J. Allergy Clin. Immunol.* 103 (1999) 362–370.
- [2] L. Li, V.A. Boussiotis, Physiologic regulation of central and peripheral T cell tolerance: lessons for therapeutic applications, *J. Mol. Med.* 84 (2006) 887–899.
- [3] C.A. Chambers, J.P. Allison, Co-stimulation in T cell responses, *Curr. Opin. Immunol.* 9 (1997) 396–404.
- [4] K.X. Luo, B.M. Sefton, Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the lck tyrosine protein kinase at the autophosphorylation site, *Mol. Cell. Biol.* 10 (1990) 5305–5313.
- [5] C. Visco, G. Magistrelli, R. Bosotti, R. Perego, L. Rusconi, S. Toma, M. Zamai, O. Acuto, A. Isacchi, Activation of Zap-70 tyrosine kinase due to a structural rearrangement induced by tyrosine phosphorylation and/or ITAM binding, *Biochemistry* 39 (2000) 2784–2791.
- [6] A.C. Chan, M. Iwashima, C.W. Turck, A. Weiss, ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain, *Cell* 71 (1992) 649–662.
- [7] M. Raab, C.E. Rudd, Hematopoietic cell phosphatase (HCP) regulates p56LCK phosphorylation and ZAP-70 binding to T cell receptor zeta chain, *Biochem. Biophys. Res. Commun.* 222 (1996) 50–57.
- [8] G. Magistrelli, R. Bosotti, B. Valsasina, C. Visco, R. Perego, S. Toma, O. Acuto, A. Isacchi, Role of the Src homology 2 domains and interdomain regions in ZAP-70 phosphorylation and enzymatic activity, *Eur. J. Biochem.* 266 (1999) 1166–1173.
- [9] C. Guntermann, D.R. Alexander, CTLA-4 suppresses proximal TCR signaling in resting human CD4(+) T cells by inhibiting ZAP-70 Tyr(319) phosphorylation: a potential role for tyrosine phosphatases, *J. Immunol.* 168 (2002) 4420–4429.
- [10] V. Di Bartolo, D. Mege, V. Germain, M. Pelosi, E. Dufour, F. Michel, G. Magistrelli, A. Isacchi, O. Acuto, Tyrosine 319, a newly identified phosphorylation site of ZAP-70, plays a critical role in T cell antigen receptor signaling, *J. Biol. Chem.* 274 (1999) 6285–6294.

- [11] M. Pelosi, V. Di Bartolo, V. Mounier, D. Mege, J.M. Pascucci, E. Dufour, A. Blondel, O. Acuto, Tyrosine 319 in the interdomain B of ZAP-70 is a binding site for the Src homology 2 domain of Lck, *J. Biol. Chem.* 274 (1999) 14229–14237.
- [12] M.L. Alegre, K.A. Frauwirth, C.B. Thompson, T-cell regulation by CD28 and CTLA-4, *Nat. Rev. Immunol.* 1 (2001) 220–228.
- [13] J.M. Choi, M.H. Ahn, W.J. Chae, Y.G. Jung, J.C. Park, H.M. Song, Y.E. Kim, J.A. Shin, C.S. Park, J.W. Park, T.K. Park, J.H. Lee, B.F. Seo, K.D. Kim, E.S. Kim, D.H. Lee, S.K. Lee, Intranasal delivery of the cytoplasmic domain of CTLA-4 using a novel protein transduction domain prevents allergic inflammation, *Nat. Med.* 12 (2006) 574–579.
- [14] L.M. Howard, A.P. Kohm, C.L. Castaneda, S.D. Miller, Therapeutic blockade of TCR signal transduction and co-stimulation in autoimmune disease, *Curr. Drug Targets Inflamm. Allergy* 4 (2005) 205–216.
- [15] S. Namiki, T. Tomida, M. Tanabe, M. Iino, K. Hirose, Intracellular delivery of glutathione S-transferase into mammalian cells, *Biochem. Biophys. Res. Commun.* 305 (2003) 592–597.
- [16] D. Klein, M.M. Ribeiro, V. Mendoza, S. Jayaraman, N.S. Kenyon, A. Pileggi, R.D. Molano, L. Inverardi, C. Ricordi, R.L. Pastori, Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets, *Biochem. Biophys. Res. Commun.* 323 (2004) 473–478.
- [17] H. Nagahara, A.M. Vocero-Akbani, E.L. Snyder, A. Ho, D.G. Latham, N.A. Lissy, M. Becker-Hapak, S.A. Ezhevsky, S.F. Dowdy, Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration, *Nat. Med.* 4 (1998) 1449–1452.
- [18] S.R. Schwarze, A. Ho, A. Vocero-Akbani, S.F. Dowdy, In vivo protein transduction: delivery of a biologically active protein into the mouse, *Science* 285 (1999) 1569–1572.
- [19] Y. Abu-Amer, S.F. Dowdy, F.P. Ross, J.C. Clohisy, S.L. Teitelbaum, TAT fusion proteins containing tyrosine 42-deleted I κ B α arrest osteoclastogenesis, *J. Biol. Chem.* 276 (2001) 30499–30503.
- [20] L.C. Sutherland, G.T. Williams, Viral promoter expression in CEM-C7 and Jurkat human T-lymphoid cell lines, *J. Immunol. Methods* 207 (1997) 179–183.
- [21] T. Moriya, K. Hiraishi, N. Horie, M. Mitome, K. Shinohara, Correlative association between circadian expression of mouse Per2 gene and the proliferation of the neural stem cells, *Neuroscience* 146 (2007) 494–498.
- [22] A. Vacca, M.P. Felli, A.R. Farina, S. Martinotti, M. Maroder, I. Screpanti, D. Meco, E. Petrangeli, L. Frati, A. Gulino, Glucocorticoid receptor-mediated suppression of the interleukin 2 gene expression through impairment of the cooperativity between nuclear factor of activated T cells and AP-1 enhancer elements, *J. Exp. Med.* 175 (1992) 637–646.
- [23] B.H. Nelson, IL-2 regulatory T cells and tolerance, *J. Immunol.* 172 (2004) 3983–3988.
- [24] D. D'Ambrosio, R. Trotta, A. Vacca, L. Frati, A. Santoni, A. Gulino, R. Testi, Transcriptional regulation of interleukin-2 gene expression by CD69-generated signals, *Eur. J. Immunol.* 23 (1993) 2993–2997.
- [25] J.M. Choi, S.H. Kim, J.H. Shin, T. Gibson, B.S. Yoon, D.H. Lee, S.K. Lee, A.L. Bothwell, J.S. Lim, Transduction of the cytoplasmic domain of CTLA-4 inhibits TcR-specific activation signals and prevents collagen-induced arthritis, *Proc. Natl. Acad. Sci. USA* 105 (2008) 19875–19880.
- [26] S. Martinez-Martinez, J.M. Redondo, Inhibitors of the calcineurin/NFAT pathway, *Curr. Med. Chem.* 11 (2004) 997–1007.
- [27] R.D. Molano, T. Berney, H. Li, P. Cattani, A. Pileggi, C. Vizzardelli, N.S. Kenyon, C. Ricordi, L.C. Burkly, L. Inverardi, Prolonged islet graft survival in NOD mice by blockade of the CD40-CD154 pathway of T-cell costimulation, *Diabetes* 50 (2001) 270–276.
- [28] C. Rastellini, A. Salam, R. Kuddus, A. Aitouche, V. Subbotin, M. Braun, R. Leach, R. Peach, J.J. Fung, T.E. Starzl, A.S. Rao, Prevention of T-cell activation by rhCTLA4-Ig and anti-CD40L monoclonal antibody results in indefinite islet allograft survival, *Transplant Proc.* 31 (1999) 1242–1243.