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## Cutting Edge: Dependence of TCR Antagonism on Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase Activity<sup>1</sup>

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*The mechanism by which antagonist peptides inhibit T cell responses is unknown. Mice deficient in Src homology 2 domain-containing protein tyrosine phosphatase (SHP-1) have revealed its importance in the negative regulation of lymphocyte signaling. We investigated a possible role for SHP-1 in T cell antagonism and demonstrate, for the first time, a substantial increase in SHP-1 activity during antagonism of CD4<sup>+</sup> T cells. Furthermore, the removal of functional SHP-1 prevents antagonism in these cells. Our data demonstrate that T cell antagonism occurs via a negative intracellular signal that is mediated by SHP-1. The Journal of Immunology, 2003, 170: 4891–4895.*

**T** cell receptor recognition of peptide/MHC is a highly sensitive interaction whereby even subtle changes in amino acids at TCR contact residues of immunogenic peptides can drastically alter the responses of the corresponding T cell (1, 2). These analog peptides are termed altered peptide ligands and are classified according to the T cell responses they evoke (2). Antagonists define a subset of altered peptide ligands that not only fail to stimulate T cell responses on their own, but also actively inhibit T cell responses to the agonist. Despite the extensive characterization of TCR antagonists, a definitive mechanism has yet to be described.

Multiple theories potentially explain how an antagonist exerts its effects on the corresponding T cell. These peptides are generally characterized as having fast dissociation rates (3, 4), and several groups have suggested that this rapid dissociation may allow antagonist ligands to successfully inhibit T cell activation by outcompeting agonists for binding to the TCR. Alternatively, antagonism is also consistent with the production of a negative signal in the cell (5–9). To examine this possibility, transgenic mice containing T cells that express two distinct TCRs (dual receptor T cells) have been used (8, 9). In these CD4<sup>+</sup> dual receptor mice the interaction of antagonist with one receptor inhibited activation signals induced by agonist

stimulation through the second receptor. Consistent with this cross-antagonism was the suggested association between Src homology 2 domain-containing protein tyrosine phosphatase (SHP-1)<sup>3</sup> and the TCR (9).

SHP-1 is an intracellular protein expressed in hematopoietic cells of all lineages, including T cells (10). Studies from natural spontaneous SHP-1 mutants have revealed a critical role for this phosphatase in the negative regulation of TCR-mediated activation of T cells, affecting the signaling threshold for both thymocytes and peripheral T cells (11–14). In this study we report a substantial increase in SHP-1 activity following antagonism of CD4<sup>+</sup> T cells. Furthermore, interference with the enzymatic activity of SHP-1 by transduction of a dominant negative mutant of the phosphatase prevented antagonism. These data collectively indicate that antagonists inhibit T cell activation by inducing a SHP-1-mediated negative signal following engagement of the TCR.

### Materials and Methods

#### *Mice, cell culture, and proliferation assays*

The 3.L2 transgenic mice (H-2<sup>b</sup>) were a gift from Dr. G. Kersh (Emory University, Atlanta, GA) and P. Allen (Washington University, St. Louis, MO). B10.A (H-2<sup>a</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed and maintained according to federal guidelines by the Emory University Department of Animal Resources. DO11.10 *me/me* transgenic mice were housed and maintained at the University of Virginia. T cell lines were generated from 3.L2 transgenic mice by stimulating splenocytes ( $1 \times 10^6$ /well) with 1  $\mu$ M hemoglobin (Hb) 64–76 (GKKVITAFNEGLK) peptide. All peptides were synthesized as previously described (8). Both proliferation and antagonism assays were conducted as previously described using gamma-irradiated B10.A splenocytes (2000 rad) in a flat-bottom 96-well plate (1, 5, 8). Antagonism is assessed using a suboptimal dose of agonist peptide on pre-pulsed APCs. Assays were harvested after 72 h and analyzed on a Matrix 96 direct beta counter (Packard Instrument, Meriden, CT).

#### *SHP-1 phosphatase assay*

T cells ( $5 \times 10^6$ ) were lysed 10 min after incubation in an antagonism assay using DCEK fibroblasts as APCs and incubated on anti-SHP-1-coated immunosorbent plates (2  $\mu$ g/ml) (Nalge Nunc International, Rochester, NY). Results were normalized to known molar values of *p*-nitrophenyl phosphate (pNPP) at 410 nm hydrolyzed by a recombinant SHP-1 protein (Upstate Biotechnology, Lake Placid, NY), where specific activity is equivalent to 1000 pmol pNPP hydrolyzed per microgram of *p*-nitrophenyl phosphate recombinant protein. A molar absorption coefficient of

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<sup>3</sup> Abbreviations used in this paper: SHP-1, Src homology 2 domain-containing protein tyrosine phosphatase; pSHP-1, phosphorylated SHP-1; Hb, hemoglobin; GFP, green fluorescent protein; pNPP, *p*-nitrophenyl phosphate.

$1.78 \times 10^4 \text{ M}^{-1} \times \text{cm}^{-1}$  was used to calculate the concentration of the *p*-nitrophenolate ion produced in the reaction. The sensitivity of the assay was confirmed by the absence of discernible phosphatase activity in lysates on both uncoated plates and plates coated with  $\alpha$ -SHP-2 Ab (data not shown).

#### Retroviral constructs and transduction of T cells

Wild-type human SHP-1 and the catalytically inactive dominant negative mutant (SHP-1 $\Delta$ P), containing a 25-aa deletion of residues 457–481, were cloned into the murine stem cell virus 2.2-internal ribosomal entry sequence-enhanced green fluorescent protein (GFP) parental vector (11, 15). Bosc 23 retroviral producer cells were transfected ( $1 \times 10^6$  cells) with 2  $\mu\text{g}$  SHP1 $\Delta$ P DNA using FuGene 6 transfection reagent (Roche, Indianapolis, IN) according to protocols of the manufacturer (16). Transduced T cells were selected 72 h after infection with 5  $\mu\text{g}/\text{ml}$  puromycin (Mediatech, Herndon, VA), and the purity of transduced T cells was confirmed by the presence of GFP.

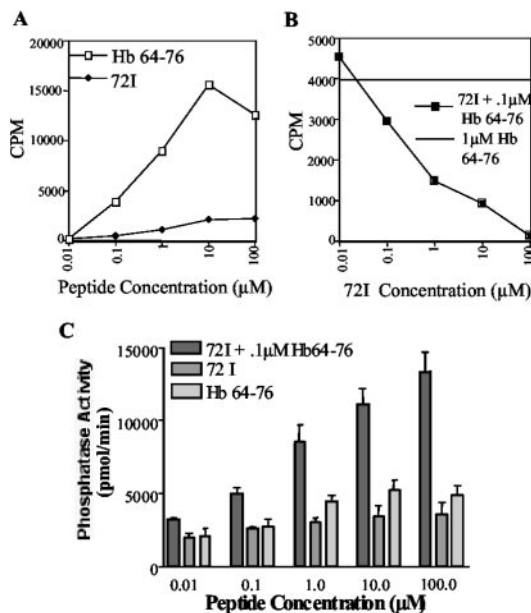
#### Immunoprecipitation and Western blots

The 3.L2 T cells ( $1 \times 10^8$ ) were lysed on ice for 20 min in 1.5 ml of lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 10 mM EDTA, 50 mM Tris (pH 8.0), 1 mM PMSF, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 10  $\mu\text{g}/\text{ml}$  aprotinin (all from Sigma-Aldrich, St. Louis, MO). Clarified lysates were added to sheep anti-mouse IgG-conjugated beads (DynaL Biotech, Lake Success, NY) precoated with 2  $\mu\text{g}$  anti-SHP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. Immunoprecipitates were resolved by 12% SDS-PAGE, transferred onto nitrocellulose (Bio-Rad, Hercules, CA), probed with HRP-conjugated anti-pTyr (Amersham Pharmacia Biotech, Piscataway, NJ), and detected using SuperSignal (Pierce, Rockford, IL). For anti-SHP-1 IgG controls, blots of SHP-1 immunoprecipitates were stripped for 15 min and then reprobbed with anti-SHP-1 (Santa Cruz Biotechnology).

## Results

### Increased active SHP-1 during antagonism

The agonist and antagonist response of 3.L2 T cells has previously been described (5, 7). The antagonist peptide 72I (N $\rightarrow$ I) failed to induce proliferation of 3.L2 T cells alone (Fig. 1A), but was able to efficiently inhibit T cell response to wild-type Hb 64–76 peptide (>90% inhibition at 100  $\mu\text{M}$  72I) (Fig. 1B). Because SHP-1 has

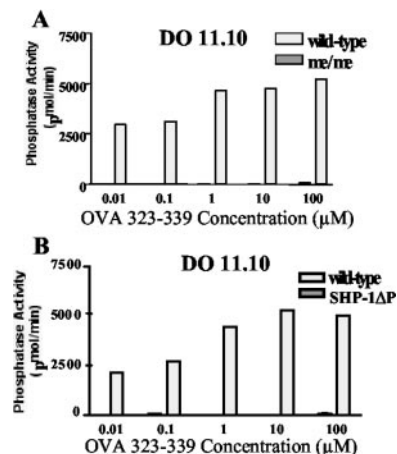


**FIGURE 1.** Increased SHP-1-associated phosphatase activity following antagonism. *A*, Proliferation of 3.L2 T cells to agonist ( $\square$ ) or antagonist ( $\blacklozenge$ ). *B*, Antagonism of 3.L2 T cells by 72I. *C*, Enzymatic activity of SHP-1 from 3.L2 lysates stimulated with agonist (Hb 64–76), antagonist alone (72I), or antagonist in the presence of agonist (0.1  $\mu\text{M}$  Hb 64–76) as measured by picomoles per minute of pNPP hydrolyzed. Data shown are the cumulative results of three independent experiments. Unstimulated 3.L2 T cells = 961 pmol/min; 0.1  $\mu\text{M}$  Hb 64–76 prepulse = 2601 pmol/min.

been implicated in playing a role during antagonism (9), its enzymatic activity was determined in response to TCR interaction with agonist or antagonist. SHP-1 was Ab-captured from lysates of 3.L2 T cells that had been exposed to peptide-pulsed APCs. The adherent DCEK fibroblasts minimized APC contamination of T cell samples, and as these cells are not of hematopoietic lineage, they lack detectable expression levels of SHP-1. SHP-1 enzymatic activity was measured by the ability of the captured phosphatase to hydrolyze pNPP. For comparison, concentrations of a recombinant SHP-1 protein were similarly assayed for phosphatase activity, and the amount of pNPP hydrolyzed at each protein concentration over time (picomoles per minute) was determined. Lysates from T cells stimulated with a submaximal level (0.1  $\mu\text{M}$  Hb 64–76) of agonist contained 2,601 pmol/min of enzymatic activity. The addition of 100  $\mu\text{M}$  antagonist (72I) increased SHP-1 enzymatic activity to 14,126 pmol/min (Fig. 1C). Interestingly, the antagonism conditions induced  $\sim$ 3-fold more SHP-1 activity than agonist peptide (5,084 pmol/min for 10  $\mu\text{M}$  Hb compared with 14,126 pmol/min for 100  $\mu\text{M}$  72I). These findings indicate that antagonism of CD4<sup>+</sup> T cells results in a dramatic and rapid increase in SHP-1 enzymatic activity.

### Abrogation of normal SHP-1 function

SHP-1 activity was eliminated by transduction of T cells with a retroviral vector encoding a dominant negative mutant SHP-1 protein (SHP-1 $\Delta$ P) that contains a 25-aa deletion in the catalytic domain to prevent substrate binding and phosphatase activity. The vector also contains enhanced GFP under the control of an internal ribosomal entry sequence to allow identification of transduced cells. Transgenic mice crossed onto the motheaten background serve as important models to determine the effect of the loss of SHP-1 activity in T cells (11, 12, 17). We chose to use the previously described DO11.10 *me/me* mice as a comparison for the efficiency of our retroviral transduction system in reducing SHP-1 activity (11). Transduction and selection of wild-type DO11.10 T cells with the SHP-1 $\Delta$ P vector resulted in 97% of the T cell population being GFP positive (data not shown). Lysates from transduced DO11.10 T cells exhibited similar levels of reduced phosphatase activity (Fig. 2B) as compared with the DO11.10 *me/me* T cells (Fig. 2A).

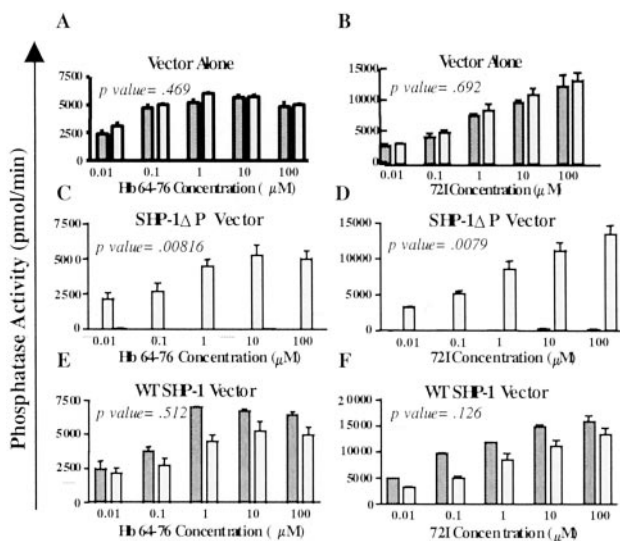


**FIGURE 2.** Comparable reduction in phosphatase activity between motheaten and transduced T cells. *A*, SHP-1 associated phosphatase activity for wild-type DO11.10 T cells (light) or DO11.10  $\times$  *me/me* T cells (shaded). *B*, SHP-1 associated phosphatase activity for wild-type DO11.10 T cells (light) or dominant negative SHP-1 (SHP-1 $\Delta$ P) transduced DO11.10 T cells.

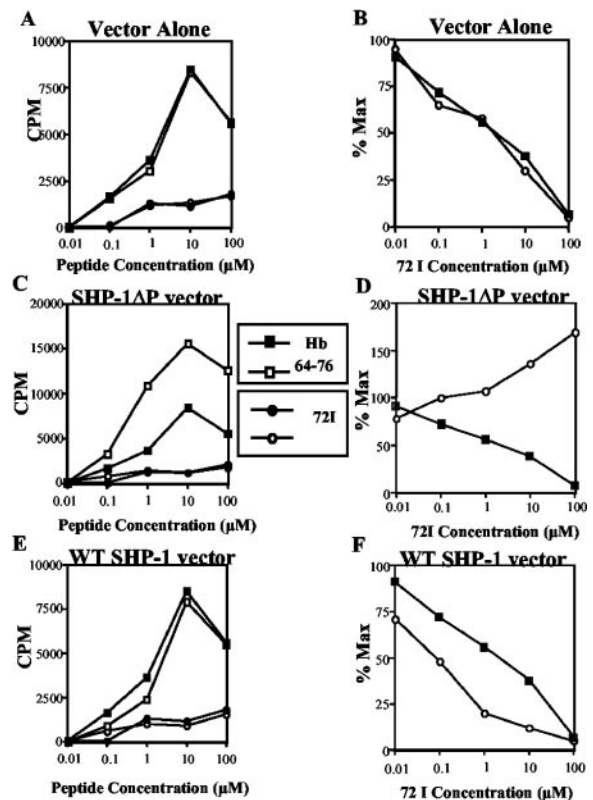
3.L2 T cells were transduced with either the SHP-1 $\Delta$ P or wild-type SHP-1 vector to investigate the role of SHP-1 during TCR antagonism. To ensure that the transduction process itself does not interfere with the functional response of the cell, 3.L2 T cells were also infected with the empty parental vector. No difference in phosphatase activity was observed in response to agonist (Fig. 3*A*) or during antagonism (Fig. 3*B*). The dominant negative SHP-1 $\Delta$ P construct abrogated phosphatase activity in response to agonist or antagonist (Fig. 3, *C* and *D*). Transduction of T cells with wild-type SHP-1 did not significantly increase phosphatase activity (Fig. 3, *E* and *F*). These data demonstrate that retroviral transduction of a catalytically inactive SHP-1 protein is an efficient method for eliminating SHP-1 activity in T cells.

#### Phenotypic changes associated with altered SHP-1 activity

The effects of the SHP-1 constructs on T cell function are shown in Fig. 4. Both control transduced and untransduced 3.L2 T cells proliferated equally well to agonist (Fig. 4*A*) and are antagonized to the same degree by 72I (Fig. 4*B*). The SHP-1 $\Delta$ P-transduced T cells are hyperresponsive to agonist peptide, as the decreased SHP-1 activity resulted in the doubling of thymidine incorporation in comparison to wild-type cells (Fig. 4*C*), consistent with previous reports observing a hyperresponsive phenotype in *me/me* T cells (11, 12). The dominant negative SHP-1 had a striking effect during TCR antagonism, as the loss of phosphatase activity correlated with a complete loss in antagonism by 72I (Fig. 4*D*). Instead of inhibiting the response in transduced T cells, 72I displayed a synergistic effect in combination with the submaximal level of agonist used to assess antagonism (Fig. 4*D*). The inability of 72I to antagonize the T cell response was not a consequence of the T cells being hyperresponsive to stimuli through the TCR, as 72I alone fails to in-



**FIGURE 3.** SHP-1 phosphatase activity following retroviral transduction of 3.L2 T cells. Lysates from wild type (light symbols) or cells transduced (shaded symbols) with the empty parental vector (*A* and *B*), SHP-1 $\Delta$ P vector (*C* and *D*), or wild-type SHP-1 vector (*E* and *F*) were stimulated with agonist (*A*, *C*, and *E*) or antagonist in the presence of 0.1  $\mu$ M Hb 64–76 prepulsed APCs (*B*, *D*, and *F*). The purity of the transduced populations was 96% or greater in all cases. Antagonist (72I) alone induced 222 pmol/min of phosphatase activity. Values of *p* were obtained by comparing the amount of phosphatase activity for wild-type lysates to that for transduced lysates from three independent experiments.

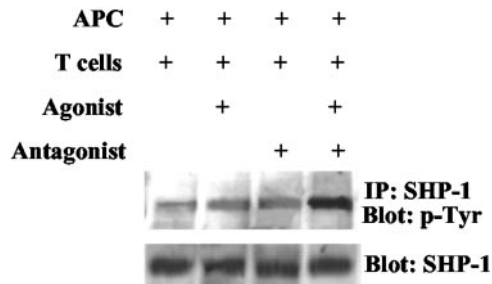


**FIGURE 4.** Altering SHP-1 changes the phenotype of antagonized 3.L2 T cells. Proliferation of wild-type (■ and ●) or transduced (□ and ○) 3.L2 T cells infected with the empty parental vector (vector alone) (*A* and *B*), SHP-1 $\Delta$ P vector (*C* and *D*), or wild-type SHP-1 vector (*E* and *F*) following stimulation with agonist or antagonist (72I) alone (*A*, *C*, and *E*) or antagonist in the presence of a 0.1  $\mu$ M Hb 64–76 prepulse (*B*, *D*, and *F*). Data shown are representative of three independent experiments.

duce proliferation in both transduced and untransduced cells (Fig. 4*C*). This finding demonstrates that the lack of SHP-1 does not endow an antagonist (72I) with agonist properties. Transduction of cells with wild-type SHP-1 caused a small but reproducible increase (20–40%) in potency of 72I during antagonism (Fig. 4*F*), suggesting that modulations in active SHP-1 may dramatically affect the extent of antagonism in CD4<sup>+</sup> T cells. Hence, the inhibition of T cell response observed under antagonism conditions is dependent on the presence of active SHP-1.

#### Phosphorylated SHP-1 (pSHP-1) in antagonized T cells

A recent report demonstrated that phosphorylation of conserved C-terminal tyrosine residues facilitated regulation of SHP-1 and increased the phosphatase activity up to 8 fold (18). Based on the substantial increase in SHP-1 activity during antagonism of CD4<sup>+</sup> T cells, we explored the possibility that more tyrosine pSHP-1 may be found in antagonized 3.L2 T cells. To determine the amount of pSHP-1 present during antagonism, the phosphatase was immunoprecipitated from T cell lysates following stimulation with agonist (Hb 64–76) alone or with the addition of 72I antagonist (Fig. 5). Analysis of tyrosine phosphorylation in SHP-1 immunoprecipitates revealed that agonist can induce a low level of pSHP-1, which correlates with the phosphatase activity observed following stimulation with Hb 64–76 (Fig. 1*C*). In contrast, more pSHP-1 protein was



**FIGURE 5.** Increased p-SHP-1 in antagonized 3.L2 T cells. SHP-1 was immunoprecipitated from 3.L2 T cells and immunoblotted for phosphotyrosine (anti-p-Tyr). All samples were lysed 10 min after stimulation.

detected in 3.L2 T cells 10 min after the addition of 100  $\mu$ M 72I to samples containing 0.1  $\mu$ M Hb prepulsed APCs. Equivalent amounts of total SHP-1 protein were detected in all lanes however, indicating that only the amount of pSHP-1 differed during antagonism. Thus, antagonism enhances tyrosine phosphorylation of SHP-1 in 3.L2 T cells.

## Discussion

Identification of the mechanism by which TCRs sense ligands of differing potency is a major goal of T cell activation studies. Significant progress toward this goal has been slowed somewhat by limited information on the biochemical events that occur following TCR interaction with antagonist peptides. A goal of our experiments was a better understanding of the molecular consequences of TCR engagement by antagonist peptide/MHC complexes. To this end we identified phosphorylated and enzymatically active SHP-1 in CD4<sup>+</sup> T cells during antagonism. This increase in phosphatase activity was observed in the 3.L2 system using an analog of Hb 64–76 (Fig. 1C) and was extended to the 3A9 system using an analog of hen egg lysozyme 48–62 (data not shown). Thus, an increase in SHP-1 activity appears to be a general property of CD4<sup>+</sup> T cells confronted with a TCR antagonist.

The phenotype observed in antagonized T cells is codependent on the presence of both agonist and antagonist (5–9). Interestingly, addition of agonist plus antagonist was also required for the greatest increase in phosphatase activity (Fig. 1C), proliferation (Fig. 4D), and tyrosine phosphorylation of SHP-1 (Fig. 5). Of particular interest was the increase in proliferation in the presence of both agonist and antagonist (Fig. 4D) following removal of SHP-1 function. It has previously been demonstrated that antagonist ligands can facilitate stabilization of the immunological synapse and therefore contribute to T cell signaling (19). It is possible that the presence of antagonist is able to facilitate signaling in response to the low level of agonist and results in the synergistic effect on proliferation.

One potential mechanism to explain antagonism is that TCR antagonists prevent receptor oligomerization and compromise the ability of signaling molecules to propagate activation of the T cell. Microscopy studies demonstrate that TCRs remain disbursed around the site of T cell/APC contact following engagement by antagonist ligands (20). It is likely that the rapid activation of SHP-1 in response to antagonist ligands (Fig. 1C) could generate an early negative signal that would prevent the formation of lipid rafts. Interestingly, a study of NK cell-mediated cytotoxicity demonstrated that engagement of killer inhibitory receptors on NK cells leads to the recruitment and activa-

tion of SHP-1 and results in a block of lipid raft polarization in the synapse as early as 1 min following conjugate formation (21). Additionally, targeting SHP-1 into lipid rafts in T cells inhibits tyrosine phosphorylation of CD3- $\zeta$  chains (22). Therefore, it is likely that a similar mechanism for T cell antagonism exists, whereby interaction of the TCR with antagonist peptide induces an early negative signal that leads to the rapid activation of SHP-1, resulting either in the cleavage of phosphates on immunoreceptor tyrosine-based activation motifs directly or in the inactivation of tyrosine kinases such as ZAP-70 or *lck*. This would prevent lipid raft polarization and accumulation of downstream signaling molecules. Experiments to identify potential receptors and substrates for SHP-1 during antagonism are ongoing.

To date, a distinct biochemical marker for T cell antagonism has not been identified. Several early signaling events have been observed to be altered in T cells following interaction with antagonist peptide. Some of these changes can include a partial pattern of tyrosine phosphorylation of the CD3- $\zeta$  chain and a lack of ZAP-70 activation (23–25). Whether these blocks in signaling have a causative effect in antagonism or are the result of an inhibitory signal are unknown. Experiments in this study have demonstrated that antagonist ligands prompt a dramatic increase in SHP-1 enzymatic activity. These findings suggest that a blockade in early signaling events observed with TCR antagonists may be the result of SHP-1 activation, and thus identify an increase in SHP-1 associated phosphatase activity as a biochemical marker of the T cell antagonist phenotype.

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