Loss of Src Homology Region 2 Domain-Containing Protein Tyrosine Phosphatase-1 Increases CD8\(^+\) T Cell-APC Conjugate Formation and Is Associated with Enhanced In Vivo CTL Function

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Loss of Src Homology Region 2 Domain-Containing Protein Tyrosine Phosphatase-1 Increases CD8+ T Cell-APC Conjugate Formation and Is Associated with Enhanced In Vivo CTL Function

Jean G. Sathish, Garry Dolton, Frances G. LeRoy, and R. James Matthews

Extensive evidence has been accumulated to implicate the intracellular protein tyrosine phosphatase, Src homology region 2 domain-containing protein tyrosine phosphatase-1 (SHP-1), as a negative regulator of TCR-signaling thresholds. Specifically, T cells from the SHP-1-deficient mouse, motheaten, exhibit a hyperproliferative phenotype when activated by cognate peptide-pulsed APCs. However, the cellular basis for this phenotype has not been fully explained. Using the intracellular fluorescent dye, CFSE, we show that a greater proportion of motheaten vs control naive CD8+ T cells undergo cell division when activated by peptide-pulsed APCs. Furthermore, there is a greater likelihood of TCRs on SHP-1-deficient vs control T cells binding to peptide/MHC ligands on APCs when using TCR down-regulation as an indirect measure of TCR engagement. In addition, T cell-APC conjugate assays provide direct evidence that a greater proportion of SHP-1-deficient T cells are capable of forming stable conjugates with APCs and this may explain, at least in part, their hyperproliferative response to TCR-triggered stimulation. The physiological relevance of the combined in vitro observations is demonstrated by the significantly enhanced in vivo expansion and CTL capacity generated in mice receiving adoptively transferred SHP-1-deficient naive CD8+ T cells when compared with control T cells. The Journal of Immunology, 2007, 178: 330–337.

A necessary prelude to T cell activation is the physical encounter and sustained contact with an APC. A prolonged contact of the T cell with the APC is essential to sustain TCR signaling and up to 20 h of such conjugation may be necessary for a naive T cell to commit to a program of full activation (1). Furthermore, the duration of stimulus received by the T cell when in contact with the APC determines its capacity to subsequently respond to homeostatic and proliferative cytokines (2). In vivo-imaging studies (3–7) also point to lengthy interactions of T cells and dendritic cells (DCs) occurring within lymph nodes (LNs) and these interactions are thought to be a prerequisite for T cell activation.

The major physical mechanism by which T cells adhere to APCs is by the binding of lymphocyte integrins on the T cell to their counterligands on the APC. Foremost among the adhesion molecules required for supporting T cell-APC conjugates is the β2 integrin, LFA-1, on T cells that binds to ICAM-1 on the APCs. T cells deficient in LFA-1 are impaired in their ability to form conjugates with APCs (8) and likewise, Abs directed against LFA-1 can block conjugate formation (9). T cell integrins normally display a state of low-binding activity toward their ligands. However, engagement of TCRs by peptide-MHC complexes on the APCs initiates signals that lead to shape changes and reorganization of the T cell actin cytoskeleton (10–13), ultimately resulting in the activation of integrins, primarily LFA-1 (14). The binding of T cell integrins to their ligands on the APC supports a stable and physical cell-cell conjugate that provides the platform for sustained signaling thus resulting in gene transcription, cytokine production, blasting, and T cell proliferation (15).

It is clear that the cellular and molecular factors influencing T cell-APC conjugate formation need to be fully understood as they are predicted to have a significant impact on T cell responsiveness and the eventual immune response. In addition to adhesion receptors, several signaling molecules including adhesion and degranulation-promoting adaptor protein (16, 17), Vav1 (18), inducible T cell kinase (19) and Src kinase-associated phosphoprotein of 55 kDa (20), and an intact actin cytoskeleton (21, 22) have been shown to be required or to assist in conjugate formation. However, negative regulators of conjugate formation have hitherto not been reported. The Src homology 2 domain containing protein tyrosine phosphatase-1 (SHP-1)-deficient motheaten mouse has been instrumental in demonstrating that SHP-1 functions to raise the signaling thresholds required for triggering through the TCR in both T cell development and in peripheral T cell activation (23, 24). To dissect the role of SHP-1 in T cells in an Ag-specific context, we have introduced the MHC class I-restricted transgenic TCR, F5 (25), into the motheaten genetic background (26). The F5 TCR recognizes a peptide derived from the influenza A virus nucleoprotein, in the context of the MHC molecule H2-Dk (27). When motheaten LN T cells bearing the F5 TCR are stimulated with cognate peptide-pulsed APCs, they exhibit a greater degree of proliferation as measured by tritiated thymidine incorporation (26)
were incubated with 1/1000 bacteriological plates in complete RPMI 1640 supplemented with 200,000 CFSE-labeled T cells to measure in vivo T cell expansion and cytotoxicity assays. To gauge T cell expansion in vivo, 3.5 × 10^6 AB cells that express H-2D^d were labeled with 0.1 μM CFSE, pulsed with peptide and incubated with 1 × 10^6 purified CD8^T cells in FACS tubes. The tubes were vortexed and conjugates allowed to form by incubation at 37°C for 90 min. Nonspecific conjugates were dispersed by vortexing and cells fixed with 4% paraformaldehyde. Cells were washed, stained with anti-CD8a and -anti-TCRβ, acquired by flow cytometry and analyzed on the electronically gated CD8^T T cell population for conjugate formation. Blocking experiments were conducted by incubating T cells with anti-CD18 integrin Ab (GAME-46; BD Pharmingen) before performing the conjugate assay.

Conjugate assay

A total of 1 × 10^6 AB cells that express H-2D^d were labeled with 0.1 μM CFSE, pulsed with peptide and incubated with 1 × 10^6 purified CD8^T cells in FACS tubes. The tubes were vortexed and conjugates allowed to form by incubation at 37°C for 90 min. Nonspecific conjugates were dispersed by vortexing and cells fixed with 4% paraformaldehyde. Cells were washed, stained with anti-CD8a and -anti-TCRβ, acquired by flow cytometry and analyzed on the electronically gated CD8^T T cell population for conjugate formation. Blocking experiments were conducted by incubating T cells with anti-CD18 integrin Ab (GAME-46; BD Pharmingen) before performing the conjugate assay.

Adhesion assay

Flat-bottom 96-well plates were coated overnight with 10 μg/ml rICAM-1 Fc chimera (Chemicon International) or 30 μg/ml fibronectin (Sigma-Aldrich) in PBS with or without 10 μg/ml anti-CD3. The plates were washed and blocked with 2.5% BSA for 2 h. Purified CD8^T cells were labeled with 1 μM of the fluorescent dye, 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (Calbiochem), resuspended in 2.5% BSA in PBS and seeded at a density of 5 × 10^6/well. Cell attachment was allowed to proceed for 30 min at 37°C followed by gentle washing of the wells with warm PBS, 2.5% BSA. Adhesion was quantified by recording fluorescence emission using a Fluostar Optima fluorescence microplate reader (BMG Labtech).

In vivo T cell expansion and cytotoxicity assays

To gauge T cell expansion in vivo, 3.5 × 10^6 positively purified CD8^T cells were injected i.v. into C57BL/6J. Rag-1^-/- recipient female mice. The recipient mice were subsequently challenged with 100 μg of NP68 peptide in IFA. Six days later, the mice were killed and spleens were harvested and homogenized. Before RBC lysis, the homogenized splenocytes were mixed with an additional reference splenocyte population to facilitate accurate quantitation of the expanded CD8^T T cells in the recipient mice. The reference population consisted of 0.5 × 10^6 reference splenocytes that had been labeled with 0.2 μM CFSE following their isolation from a C57BL/6J mouse. The mixed splenocyte populations were stained with anti-CD8 and analyzed by flow cytometry. The percentage of adoptively transferred and expanded CD8^T T cells was assessed by comparison with a defined number of the CFSE-labeled reference splenocytes. To measure in vivo cytotoxicity, 2–3 × 10^6 positively purified CD8^T cells were injected i.v. into sublethally (650 cGy) irradiated C57BL/6J recipient female mice. Reference recipient mice received no T cells. In parallel, 4 × 10^5 mature DCs, pulsed with 5 μM NP68 peptide, were injected i.p. into recipient mice. Six days later, splenocyte targets from naive mice were labeled with either 2 or 0.2 μM CFSE. Splenocyte targets labeled with 0.2 μM CFSE were pulsed for 1 h with 5 μM NP68 while the proliferation index is defined as the average number of divisions undergone by those T cells in the starting population that did divide.

Flow cytometry

Cells were washed and incubated with the indicated conjugated Abs in stain buffer (Cell Wash, 2% BSA, and 2 mM EDTA) for 30 min on ice. Conjugated Abs were obtained as follows; anti-CD8a was from Caltag Laboratories, anti-TCRβ from BD Biosciences and stained with anti-Vp11 FITC (KT-11) and anti-CD8α in stain buffer for 30 min on ice followed by washing. The cells were acquired on the flow cytometer (FACSCalibur; BD Biosciences) and analyzed by CellQuest Software.

TCR down-regulation assay

Purified CD8^T cells were incubated with AB cells pulsed with a saturation (10 μM) concentration of NP68 peptide in FACS tubes for 4 h. At the end of the incubation, the cells were washed with Cell Wash (BD Biosciences) and stained with anti-Vp11 FITC (KT-11) and anti-CD8α in stain buffer for 30 min on ice followed by washing. The cells were acquired on the flow cytometer and TCR down-regulation assessed on the electronically gated CD8^T T cell population. For anti-CD3-induced down-regulation, T cells were incubated with a titration of anti-CD3αβ ranging from 1.25 to 20 μg/ml on ice for 30 min followed by cross-linking with streptavidin (Pierce Biotechnology) for an additional 30 min on ice. The cells were then incubated at 37°C for 45 min and TCR down-regulation stopped by addition of ice-cold Cell Wash (containing NaN3). Cells were then stained with anti-CD8a and -anti-TCRβ, acquired on the flow cytometer and TCR down-regulation was measured as indicated above.

Conjugate assay

Materials and Methods

Mice

C57BL/6J mice heterozygous at the motheaten locus (C57BL/6J me/me) were originally obtained from Dr. L. Shultz at The Jackson Laboratory. The F5-transgenic TCR was introduced into the motheaten genetic background as described in Ref. 29 and motheaten and littermate control mice for experimentation were obtained from litters arising from pairings between C57BL/6J me/me and F5 homozygous me/mice. The F5 TCR uses the Vα4 and Vβ11 gene segments of the TCR α- and β-chain genes, respectively. Motheaten and control mice were sacrificed between 9 and 13 days postpartum and LNs (inguinal, axillary, brachial, and submandibular) harvested. C57BL/6J mice purchased from The Jackson Laboratory and C57BL/6J. Rag-1^-/- mice provided by Dr. F. Powrie (Sir William Dunn School of Pathology, Oxford University, Oxford, U.K.) were maintained as breeding colonies in our animal facility. All animal experimentation was conducted in accordance with the U.K. Animal (Scientific Procedures) Act 1986 under Project Licenses PPL 30/1715 and 30/2266.

Cells and cell cultures

The mouse B cell line, AB, was generated from H-2ab bone marrow-derived B cells transfected with Abelson leukemia virus and was a gift from Prof. E. Simpson (Imperial College, London, U.K.; Ref. 29). This cell line was maintained in complete DMEM supplemented with 50 μM 2-ME. Bone marrow-derived DCs were grown as described in Ref. 29. Briefly, bone marrow was flushed from the tibia of C57BL/6J mice and cultured on bacteriological plates in complete RPMI 1640 supplemented with 200 μM recombinant mouse GM-CSF (Sigma-Aldrich). At days 5 and 8, half the medium was replaced and 100 μM recombinant mouse GM-CSF was added. At day 10, 1 μg/ml LPS was added and 24 h later, mature DCs were pulsed with 5 μM NP68 and used experimentally. Purified CD8^T cells were obtained by negative depletion of CD4^+ T cells, B cells, and myeloid cells with CD4, CD11b, and B220 microbeads (Miltenyi Biotec) or where indicated by positive purification with CD8 microbeads (Miltenyi Biotec) and used in the experiments.

T cell proliferation assay

For cell division measured by CFSE dilution, purified CD8^T cells were labeled with 2 μM CFSE (Molecular Probes) for 10 min in HBSS at 37°C. Labeling was stopped by addition of FCS and cells were washed and resuspended in complete DMEM. A total of 1 × 10^6 CFSE-labeled T cells were incubated with 1 × 10^6 irradiated, peptide-pulsed AB cells for 48 h. At the end of the stimulation, T cells were stained with anti-CD8a and acquired by flow cytometry. T cell proliferation was measured by CFSE fluorescence dilution of the electronically gated CD8^T cell population. CFSE profiles were analyzed using the proliferation software program (FlowJo software program (Tree Star). The division index indicates the average number of divisions that a T cell in the starting population underwent and the proliferation index is defined as the average number of divisions undergone by those T cells in the starting population that did divide.

Flow cytometry

Cells were washed and incubated with the indicated conjugated Abs in stain buffer (Cell Wash, 2% BSA, and 2 mM EDTA) for 30 min on ice. Conjugated Abs were obtained as follows; anti-CD8α was from Caltag Laboratories, anti-TCRβ from BD Biosciences and stained with anti-Vp11 FITC (KT-11) and anti-CD8α in stain buffer for 30 min on ice followed by washing. The cells were acquired on the flow cytometer (FACSCalibur; BD Biosciences) and analyzed by CellQuest Software.
Results

A higher frequency of motheaten T cells undergo cell division upon stimulation

Previous studies have demonstrated that SHP-1-deficient motheaten T cells hyperproliferate when stimulated by peptide-bearing APCs (23, 24, 26). In each of these studies, a consistent finding was that motheaten T cells exhibited a higher proliferative capacity at all the doses of peptide tested. Although [3H]thymidine incorporation is a sensitive measure of proliferative activity, it does not distinguish between the possibility that a limited number of motheaten T cells underwent multiple rounds of cell division or that a larger proportion of the initial SHP-1-deficient T cell population entered into cell division. Both these processes could account for the increased [3H]thymidine incorporation by motheaten T cells when compared with control T cells. Labeling T cells with the intracellular fluorescent dye, CFSE, allows one to address this particular question. Therefore, we used CFSE labeling to track cell division in motheaten and control T cells. As shown in Fig. 1, when control T cells were stimulated by APCs pulsed with 10 μM NP68, 32% of the initial CD8+ T cell population underwent cell division. However, the same stimulus elicited an ~2-fold increase (62%) in the proportion of motheaten T cells that entered into cell division. An increase in the concentration of cognate peptide used to stimulate the T cells did not significantly alter the relative proportions of motheaten and control T cells induced to divide. A comparison of the proliferation indices for motheaten and control T cells suggested that once entered into cell cycle, the differences in cell division were maintained in subsequent divisions (Table I). Finally, neither motheaten nor control T cells underwent spontaneous cell division when incubated with unpulsed APCs (result not shown).

The increased entry into cell division (Fig. 1) was consistent with a lowered TCR-signaling threshold in the absence of SHP-1 but assumed equivalent engagement of TCR on motheaten and control T cells. It was therefore necessary to explore whether there was an altered likelihood of motheaten vs control T cells encountering Ag presented by MHC class I on the APCs. It has been observed previously that in vitro TCR engagement by MHC/peptide complexes or cross-linking with Abs triggers down-regulation of the TCR from the T cell surface (31). Accordingly, TCR down-regulation can be used as a valid indicator of Ag encounter by a T cell (21, 32). Therefore, we measured TCR down-regulation to ascertain whether motheaten T cells experienced an altered frequency of Ag encounter. It is important to note that equal proportions of motheaten and control CD8+ T cells purified from the LNs expressed the F5 TCR as assessed by staining with KT11, a mAb between motheaten and control T cells in terms of number of subsequent divisions are less pronounced (Table I). Finally, neither motheaten nor control T cells underwent spontaneous cell division when incubated with unpulsed APCs (result not shown).

The increased entry into cell division (Fig. 1) was consistent with a lowered TCR-signaling threshold in the absence of SHP-1 but assumed equivalent engagement of TCR on motheaten and control T cells. It was therefore necessary to explore whether there was an altered likelihood of motheaten vs control T cells encountering Ag presented by MHC class I on the APCs. It has been observed previously that in vitro TCR engagement by MHC/peptide complexes or cross-linking with Abs triggers down-regulation of the TCR from the T cell surface (31). Accordingly, TCR down-regulation can be used as a valid indicator of Ag encounter by a T cell (21, 32). Therefore, we measured TCR down-regulation to ascertain whether motheaten T cells experienced an altered frequency of Ag encounter. It is important to note that equal proportions of motheaten and control CD8+ T cells purified from the LNs expressed the F5 TCR as assessed by staining with KT11, a mAb between motheaten and control T cells in terms of number of subsequent divisions are less pronounced (Table I). Finally, neither motheaten nor control T cells underwent spontaneous cell division when incubated with unpulsed APCs (result not shown).

### Table I. Quantitative analysis of motheaten and control CD8+ T cell proliferation in vitro

<table>
<thead>
<tr>
<th>Culture</th>
<th>NP68 (pM)</th>
<th>Division Indexa</th>
<th>Proliferation Indexa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Motheaten</td>
<td>10</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.4</td>
<td>1.4</td>
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</table>

a Measure of the average number of divisions that a T cell in the starting population underwent.
b Measure of the average number of divisions undergone by a T cell that did divide.
Increased APC-T cell conjugate formation by motheaten T cells that is LFA-1 dependent

The enhanced down-regulation of TCR detected on motheaten vs control T cells when presented with APC-bearing peptide but not with soluble anti-CD3 Ab was suggestive of an increased ability of motheaten T cells to engage APCs. Therefore, we predicted that motheaten T cells may have an altered capacity to form conjugates with peptide-pulsed APCs and performed conjugate assays to test this hypothesis. The AB cells were labeled with CFSE to facilitate identification of the T-B cell conjugates. Fig. 4 shows that when control CD8+ T cells were incubated with AB cells pulsed with NP68 peptide at a concentration of 10 pM, the percentage of T cells that formed stable conjugates increased from a baseline of ~6% to that of 28%. However, motheaten T cells showed a significantly higher frequency (57%) of conjugate formation when presented with the same concentration of peptide. The result of this experiment demonstrated that the lack of SHP-1 in the motheaten T cells confers an enhanced capacity to engage and form stable conjugates with the APC and this is manifested as an increased frequency of T cell-APC conjugates.

Conjugate formation is supported by the binding of T cell integrins to counterligands on the APC. LFA-1 is the critical integrin that mediates T-B cell conjugation (8) and it was therefore important to investigate whether the motheaten T cells retained a similar dependence on LFA-1 engagement for conjugate formation. Conjugate formation was therefore examined following blocking of LFA-1-ICAM-1 interaction using an anti-LFA-1 Ab. Fig. 4 shows that when motheaten and control T cells were incubated with unpulsed APCs and anti-LFA-1 Ab, the basal level of conjugate formation was reduced from 6 to 2% for both populations of T cells.

To distinguish between these two possibilities, we measured TCR down-regulation following cross-linking with soluble anti-CD3 Ab. This approach eliminates any contribution made to TCR down-regulation by the interaction of the APC with the T cell. The anti-CD3 Ab is expected to cross-link TCRs on motheaten and control T cells to an equal degree. As demonstrated in Fig. 3, both motheaten and control T cells revealed equivalent TCR down-regulation after 45 min of cross-linking the TCR/CD3 complex with a range of concentrations of anti-CD3 Ab. This indicates that, following direct CD3 cross-linking at least, the intrinsic process of TCR internalization proceeds to a similar degree in motheaten and control T cells.

### Increased number of LFA-1-dependent APC-T cell conjugates formed by motheaten T cells

To determine whether the motheaten T cells retained a similar dependence on LFA-1 engagement for conjugate formation, conjugate formation was examined following blocking of LFA-1-ICAM-1 interaction using anti-LFA-1 Ab. Fig. 4 shows that when motheaten and control T cells were incubated with unpulsed APCs and anti-LFA-1 Ab, the basal level of conjugate formation was reduced from 6 to 2% for both populations of T cells.

### FIGURE 4

Increased number of LFA-1-dependent APC-T cell conjugates formed by motheaten T cells. Purified motheaten and control CD8+ T cells expressing the F5 TCR were incubated with or without anti-CD18 Ab before incubation for 90 min at 37°C with AB cells labeled with 0.1 μM CFSE and left unpulsed or pulsed with 100 pM NP68 peptide. Nonspecific conjugates were dispersed by vortexing and conjugates stained with anti-CD8 TC. APC-T cell conjugates were identified by flow cytometry on the electronically gated CD8+ T cell population based upon increased FSC and CFSE fluorescence of conjugated cells. The percentage of naive CD8+ T cells that have formed conjugates with the APCs is indicated in each dot plot. Results are representative of three independent experiments.
cells displayed an equivalent expression of LFA-1, VLA-4, and VLA-5. We conclude from these results that the differences in conjugate formation between motheaten and control T cells cannot be attributed to differential integrin expression. Under resting conditions, T cell integrins do not exhibit a significant level of binding activity to their ligands. However, upon activating the T cell through TCR engagement, integrin binding to ligand is up-regulated by a modulation of integrin affinity and avidity (14). Although the expression levels of surface integrins are similar in the motheaten and control T cells, it is possible that TCR-induced activation might result in enhanced integrin activation in the motheaten T cells. This may then lead to increased conjugate formation. To examine this possibility, we fluorescently labeled motheaten and control T cells and performed adhesion assays. T cells were allowed to adhere onto wells coated with the LFA-1 ligand, ICAM-1, either alone or in conjunction with anti-CD3 (Fig. 5B). Alternatively, T cell adhesion to wells coated with the VLA-4 and 5 ligand, fibronectin, either singly or in conjunction with anti-CD3, was analyzed (Fig. 5B). The combined results of these experiments indicated that motheaten and control T cells adhere to plate-bound integrin ligands to an equal degree, both basally and following anti-CD3 stimulation.

**Enhanced in vivo expansion and cytolysis by motheaten T cells**

The increased conjugation of motheaten CD8+ T cells to APCs is likely to provide the basis for the enhanced proliferative capacity of these T cells that is observed in vitro. However, the physiological requirements placed upon a naive CD8+ T cell for expansion in vivo are more complex and include entry into peripheral LNs leading to encounter with Ag-presenting DCs. Hence, it was important to explore whether loss of SHP-1 in CD8+ T cells also translates into enhanced expansion in vivo. The severe autoreactivity and early morbidity associated with the motheaten phenotype (33) precludes any direct in vivo experimentation. Therefore, to address the issue of in vivo relevance, equivalent numbers of naive F5 CD8+ T cells purified from either motheaten or littermate control mice were adoptively transferred into irradiated recipient mice that had been pulsed or not with NP68 peptide and differentially labeled with CFSE. An additional 24 h later, the proportions of differentiated CFSE-labeled cells were assessed as a measure of in vivo CTL activity. Fig. 6B reveals that in vivo CTL activity was readily detected in recipient mice receiving wild-type T cells but the level of killing was significantly increased (84 vs 56%) in mice receiving motheaten T cells. In reference recipient mice that received no adoptively transferred T cells, equivalent numbers of each population of target splenocytes were detected. Importantly, these results demonstrate that the increased in vivo expansion of motheaten CD8+ T cells also extends to an elevated cytolysis of target cells.

Furthermore, blocking LFA-1 binding resulted in a similar complete inhibition of conjugate formation for both the motheaten and control T cells following activation with B cells pulsed with 10 pM NP68 peptide. It is therefore apparent from this result that motheaten T cells retain an equivalent dependence on LFA-1 for conjugate formation when compared with control T cells.

**Equivalent adhesion of motheaten and control T cells to ICAM-1 and fibronectin**

Given the dependence upon LFA-1 for motheaten and control T cells to form conjugates, it is possible that the increased numbers of motheaten T cells forming conjugates could be due to a higher expression of LFA-1 on the surface of motheaten T cells. To test this possibility, the expression of the α-chain of LFA-1 and related T cell integrins, VLA-4 and VLA-5, was examined by flow cytometry. As shown in Fig. 5A, both the motheaten and control T cell populations CD8+FITC conjugates, anti-CD11a, anti-CD49d, and anti-CD49e. Integrin expression was assayed by flow cytometry on electronically gated populations CD8+ T cell populations. Histograms depicting integrin expression for motheaten and control T cells are shown and are representative of three independent experiments. Filled and bold-line histograms represent control and motheaten T cell population, respectively. Staining with isotype-matched controls is indicated by the open histograms. B, Purified motheaten and control CD8+ T cells expressing the F5 TCR were labeled with 1 μM BCECF/AM and seeded at a density of 5 × 106/well precoated with 10 μg/ml rICAM-1 Fc chimera or 30 μg/ml fibronectin with or without 10 μg/ml anti-CD3. Cells were incubated for 30 min at 37°C followed by gentle washing and adhesion assessed as recorded fluorescence. Bar graphs show the mean percentages of bound T cells in each of the four experimental conditions. Error bars represent SDs. Results are representative of five independent experiments.
anti-CD8 PE and analyzed by flow cytometry. The relative percentage of C57BL/6J mouse. The mixed splenocyte populations were stained with /H11003 were harvested and before RBC lysis, the homogenized splenocytes were in independent experiments. killed is indicated in each histogram. Results are representative of three rations respectively. The percentage of NP68-pulsed splenocyte targets assessed by flow cytometry. The marker regions, M1 and M2, on the his- were injected i.v. into the recipient mice. Spleens were harvested after an pleted for 1 h with 5 /H9262 control CD8 /H11001 adoptedly transferred and expanded CD8 /H11003 was assessed by com-parison with a defined number of the CFSE-labeled reference splenocytes and this is indicated in each histogram. Results are representative of three independent experiments. B. A total of 2–3 × 10⁶ purified motheaten or control CD8⁻ T cells expressing the F5 TCR were injected i.v. into irradiated C57BL/6J recipient mice. A reference recipient mouse group re-ceived no T cells. In parallel, 4 × 10⁶ mature DCs pulsed with 5 μM NP68 peptide were injected i.p. into the recipients. Six days later, splenocyte targets were differentially labeled with either 2 or 0.2 μM CFSE. The population of T cells labeled with the lower concentration of CFSE was pulsed for 1 h with 5 μM NP68 before equal numbers of both populations were injected i.v. into the recipient mice. Spleens were harvested after an additional 24 h and the proportions of differentially CFSE-labeled cells assessed by flow cytometry. The marker regions, M1 and M2, on the his-tograms represent the NP68-pulsed and unpulsed splenocyte target popu-lations respectively. The percentage of NP68-pulsed splenocyte targets killed is indicated in each histogram. Results are representative of three independent experiments.

Discussion

In this study, we have addressed the cellular mechanism that underlies the hyperproliferative phenotype of SHP-1-deficient motheaten T cells and examined its in vivo relevance. The increased proliferation, as measured by thymidine incorporation, of SHP-1-deficient T cells upon Ag receptor stimulation could have resulted from an increase in the proportion of T cells entered into cell division, an increase in the number of divisions, or a combination of these possibilities. To distinguish between the different possibilities, the labeling of motheaten and control T cells with the intracellular fluorescent dye, CFSE (34), has allowed us to analyze the proportion of each population entered into cell division following in vitro antigenic stimulation. The major conclusion from these experiments is that loss of SHP-1 results in an ~2-fold increase in the percentage of naive CD8⁺ T cells entering into cell division when exposed to APCs bearing the appropriate MHC/peptide complex. Once committed to the first cell division, the subsequent number of cell divisions undertaken by motheaten and control T cells appears similar.

The question therefore shifts as to why the probability of entry into cell cycle is significantly enhanced for individual SHP-1-deficit T cells following exposure to Ag-bearing APCs. Previous studies have suggested that TCR-signaling thresholds are lowered in the absence of SHP-1 (26). However, these conclusions have assumed an equivalent engagement of TCR on motheaten and control T cells by appropriate MHC complexes on the surface of the APC. Using TCR down-regulation to interrogate the history of F5 TCR engagement by H-2Db/NP68 ligand complexes on B cells, we noted a significantly increased likelihood of TCR engagement on motheaten vs control T cells. Hence, the heightened proliferative responses of motheaten T cells in response to stimulation with cognate peptide-pulsed APCs most likely derive in part from the increased probability of TCR engagement. As a control, cross-linking with soluble anti-CD3 Ab demonstrated that there was no intrinsic difference between motheaten and control T cells for TCR internalization. However, previous data from others (35, 36) and our own group (37) has demonstrated that SHP-1-deficient T cells hyperproliferate in response to anti-CD3 cross-linking. The implication from these combined results is that while SHP-1 can influence the degree of engagement of TCR with its counterligand on the surface of the APC, the effects of SHP-1 on T cell responsiveness cannot be limited solely to the enhanced engagement of TCR but must also influence TCR-signaling thresholds.

When a T cell physically encounters an Ag-bearing APC, a concomitant shape change is induced in the T cell that causes the T cell to spread on the APC surface thus increasing the surface area of contact (38). In addition, relaxation of T cell rigidity, a process regulated by ezrin-radixin-moesin proteins, facilitates efficient T cell-APC conjugate formation (39). Both the initial degree of rigidity and capacity for spreading of the T cell membrane over the surface of the APC are dependent upon the cortical actin cytoskeleton and intriguingly a connection between SHP-1 and the regulation of cytoskeletal processes has begun to emerge (40, 41). It is therefore plausible that an effect of SHP-1 activity on actin polymerization and cytoskeleton remodeling may underlie its ability to affect T cell adhesion to APCs. One possibility is that loss of SHP-1 induces changes in the lymphocyte actin cytoskeleton that enhances the capacity of motheaten T cells to scan the surface of neighboring APCs. This would increase the likelihood of TCRs on the surface of motheaten T cells engaging with MHC/peptide ligands on the APCs.

The enhanced probability of TCR engagement observed for SHP-1-deficient T cells is subsequently manifested in the increased capacity of these T cells to form stable cellular conjugates with the APC. Stable T cell-APC conjugate formation results from TCR-triggered increases in the binding activity of LFA-1. We therefore examined whether increases in LFA-1-binding activity, as measured by T cell adhesion to the plate-bound LFA-1 ligand, ICAM-1, could be detected on motheaten T cells following anti-CD3 stimulation. Interestingly, while conjugate formation by SHP-1-deficient and control T cells could be shown to be critically dependent upon LFA-1, no significant differences in the level of expression or binding capacity of LFA-1 to ICAM-1 were detectable when comparing motheaten and control T cells following anti-CD3 stimulation. Therefore, it is possible that the SHP-1 effect on T cell-APC conjugation does not influence the signals that link TCR triggering to LFA-1 activity.
An effect of SHP-1 on cell-cell adhesion has also been demonstrated for NK cells whereby SHP-1 activity can induce a remarkable suppression of NK cell cytolysis of targets. When SHP-1 is recruited and activated by an inhibitory killer Ig-like receptor, on the NK cell line, YTS, conjugates formed between the NK cell and target cells are dramatically reduced (42). Similar to our T cell–APC conjugate system, NK cell-target cell conjugate formation is inhibited by the blocking of LFA-1 binding (42). The inhibition of NK cell killing also appears to be mediated via disruption of actin cytoskeleton remodeling whereby the guanine nucleotide exchange factor, Vav1, has been implicated as a direct substrate of SHP-1 (43, 44). Vav1 is also a candidate substrate for SHP-1 in T cells although the results presented in this study imply that there may be at least one other substrate of SHP-1 that influences the ability of individual TCRs on the T cell surface to engage MHC/peptide complexes on the APC.

The overall significance of T cell-APC conjugate formation is underscored by a number of in vivo imaging studies (3–6) that have revealed a correlation between T cell activation leading to proliferation and the ability of T cells to establish long-lasting stable contact with DCs. Further evidence for the critical relevance of T cell-APC conjugate formation also derives from recent observations (45, 46) indicating that T reg cells may affect the immune response by reducing the capacity for contact formation between DCs and effector T cells. Clearly, a more comprehensive understanding of the cellular and molecular requirements for T cell-APC conjugate formation is needed. The increased percentage of conjugates formed with moth eaten CD8+ T cells would be predicted to result in increases in the number of T cells capable of IL-2 synthesis and secretion as stable interactions between T cells and APCs appear to be a prerequisite for IL-2 synthesis to proceed. In an elegant in vitro imaging study, whereby transgenic CD4+ T cells expressing an IL-2 promoter GFP reporter construct were allowed to engage Ag-pulsed DCs, it was demonstrated that only a subset of those T cells that had prolonged and stable interactions with the DCs expressed IL-2 (47). Transient interactions between T cells and DCs were only sufficient for the expression of IL-2 in T cell activation markers such as the α-chain of the IL-2R. We envisage that the greater number of stable conjugates formed with moth eaten CD8+ T cells results in a higher proportion of T cells becoming IL-2 producers thereby increasing the recruitment of T cells into the proliferating population. Indeed, we have shown previously that a population of motheaten vs control T cells secreted more IL-2 when stimulated with peptide-pulsed APCs (26). The results reported here have established that motheaten T cells can more readily form conjugates with the APC. However, once conjugates have been allowed to form, it remains to be established whether motheaten T cells retain a further increased capacity for synthesizing IL-2 when compared with control T cells.

The enhanced capacity for conjugate formation leading to increased in vitro proliferative expansion displayed by SHP-1-deficient CD8+ T cells is noteworthy. However, demonstrating effects in vitro does not necessarily equate to how T cells might respond in vivo whereby additional constraints are imposed. Indeed, a naive CD8+ T cell must have the capacity to expand in peripheral LN, differentiate into a mature CTL, and traffic from the Ag-draining LN to peripheral tissues before eventually lysing the appropriate target cell. Hence, our observations demonstrating enhanced in vivo killing by a population of SHP-1-deficient naive CD8+ T cells following their adoptive transfer and in vivo expansion are especially significant because they establish for the first time that loss of SHP-1 increases the proliferative capacity of T cells in an in vivo context extending to an elevation of CTL killing, the ultimate physiological challenge for a CD8+ T cell.

In conclusion, we provide evidence that SHP-1 negatively regulates the formation of conjugates between CD8+ T cells and APCs. This effect of SHP-1 activity must account, at least in part, for the hyperresponsive phenotype of motheaten T cells observed in different parameters of T cell function that have been measured (26). Importantly, the enhanced in vivo cytotoxicity of naive SHP-1-deficient T cells highlights the validity of targeting SHP-1 expression by small interfering RNA and pharmacological approaches to potentially boost human CD8+ T cell function.

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Disclosures

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References


