Cutting Edge: Rescue of Pre-TCR but Not Mature TCR Signaling in Mice Expressing Membrane-Targeted SLP-76

Natalie A. Bezman, Rebecca G. Baker, Laurie E. Lenox, Martha S. Jordan and Gary A. Koretzky

J Immunol 2009; 182:5183-5187; doi: 10.4049/jimmunol.0802176
http://www.jimmunol.org/content/182/9/5183

References
This article cites 21 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/182/9/5183.full#ref-list-1

Why The JI? Submit online.
- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Rescue of Pre-TCR but Not Mature TCR Signaling in Mice Expressing Membrane-Targeted SLP-76

Natalie A. Bezman, Rebecca G. Baker, Laurie E. Lenox, Martha S. Jordan, and Gary A. Koretzky

SLP-76 (Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa) organizes signaling from immunoreceptors, including the platelet collagen receptor, the pre-TCR, and the TCR, and is required for T cell development. In this study we examine a mouse in which wild-type SLP-76 is replaced with a mutant constitutively targeted to the cell membrane. Membrane-targeted SLP-76 (MTS) supports ITAM signaling in platelets and from the pre-TCR. Signaling from the mature TCR, however, is defective in MTS thymocytes, resulting in failed T cell differentiation. Defective thymic selection by MTS is not rescued by a SLP-76 mutant whose localization is restricted to the cytosol. Thus, fixed localization of SLP-76 reveals differential requirements for the subcellular localization of signaling complexes downstream of the pre-TCR vs mature TCR. The Journal of Immunology, 2009, 182: 5183–5187.

hymocyte development is defined by a sequence of differentiation stages characterized by the expression of cell surface molecules. CD4/CD8 double negative (DN) thymocytes progress through DN1 (c-kit+/CD25−), DN2 (c-kit−/CD25+) and DN3 (c-kit−/CD25−) stages independently of the TCR (1). At the DN3 stage, signaling from the pre-TCR results in transition through the DN4 stage (c-kit−/CD25−) to CD4/CD8 double positive (DP) cells in a process known as β-selection (2). DP thymocytes express the mature αβTCR and both CD4 and CD8. Moderate signals from the TCR promote positive selection, whereas strong TCR signaling results in negative selection (3). How signals from the pre-TCR and then the TCR direct maturation and selection in thymocytes and activation of mature T cells is an area of intense investigation. Although common signaling elements direct each response, how signals are integrated and translated into the appropriate outcome is not fully understood.

Given the critical role of SLP-76 relocalization to LAT, we hypothesized that SLP-76 targeted to LAT would support TCR signaling. We generated a chimeric molecule containing the membrane and lipid raft-targeting sequences of LAT fused to full-length SLP-76. Membrane-targeted SLP-76 (MTS) localized to membrane lipid raft domains and restored TCR signaling in SLP-76-deficient Jurkat T cells. We predicted that MTS would also support T cell development and function in a genomic knockin MTS mouse in which LCP2 (gene encoding SLP-76) is modified with the LAT membrane-targeting sequence. Surprisingly, MTS did not partition into lipid rafts in thymocytes despite localization to cell membranes. Unlike SLP-76+/− thymocytes that arrest at the DN3 stage, MTS thymocytes successfully differentiate into DP cells. Development is impaired at the DP to single positive (SP) transition, and failure of the MTS to support the generation of SP cells is associated with defects in TCR signaling. In contrast, signaling via the

1Abramson Family Cancer Research Institute, Children’s Hospital of Philadelphia, Department of Pathology and Laboratory Medicine, and Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104

Received for publication June 17, 2008. Accepted for publication March 6, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2N.A.B. and R.G.B. contributed equally to this work.

3Address correspondence and reprint requests to Dr. Gary A. Koretzky, University of Pennsylvania, 415 Biomedical Research Building II/III, 421 Curie Boulevard, Philadelphia, PA 19104. E-mail address: Koretzky@mail.med.upenn.edu

4Abbreviations used in this paper: DN, double negative; CVX, convulxin; DP, double positive; Gads, Grb-2-related adapter protein downstream of Shc; GPVI, glycoprotein VI; LAT, linker of activated T cells; MTS, membrane-targeted SLP-76; PLC, phospholipase C; PTK, protein tyrosine kinase; SLP-76, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa; SP, single positive; WT, wild type.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/S2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802176
ITAM-containing platelet collagen receptor is completely intact in MTS mice. These studies demonstrate a genetic approach to separate the requirements for SLP-76 localization during thymic development and illustrate how different cell types may use adapter protein localization to direct biological responses.

Materials and Methods

Generation of MTS knockin mice

Base pairs 1–105 of murine LAT cDNA were fused to LCP2 exon 1 and intron 1 DNA sequences. The LCP2 ATG was mutated, and the resulting fragment was cloned into the pPNT-FRT vector (M. Kahn, University of Pennsylvania, Philadelphia, PA) adjacent to the FRT-flanked neoR cassette. The targeting construct was electroporated into R1 embryonic stem cells, screened by Southern blotting, and injected into B6 × 129 blastocysts. The neoR cassette was excised by breeding to FLP mice (The Jackson Laboratory).

Genetic reconstitution of MTS/MTS fetal liver

Fetal liver was retrovirally transduced with MIGR1 (9). Recipient mice were analyzed 6–8 wk postreconstitution. All mice were housed under pathogen-free conditions at the University of Pennsylvania Animal Care Facility and used in accordance with National Institutes of Health guidelines and approved protocols.

Subcellular fractionation

Cytosol and membranes were purified as described (10). Thymocytes were sheared and nuclei pelleted by low speed centrifugation. Cytosol was isolated as supernatant following ultracentrifugation, and Nonidet P-40 solubilization isolated cell membranes.

Purification of lipid rafts

Thymocytes were lysed in MES buffer with 1% Triton and resuspended in MES with 40% sucrose. Sucrose solutions of 30 and 5% were overlaid, and lysates were submitted to overnight ultracentrifugation (44,000 × g at 4°C). Three hundred-microliter fractions were taken from the top and analyzed.

Ca2+ flux

Thymocytes were loaded with 2 μg/ml Indo-1, coated with biotinylated anti-CD3 (2G11) and anti-CD4 (RMA-4), and stained for CD4 (GK1.5) and CD8 for 30 min at 30°C. Ca2+ flux was triggered with streptavidin (12.5 μg/ml) and ionomycin (2 μg) as a positive control. Ca2+ release was measured by Indo-1 fluorescence.

Immunofluorescent microscopy

Thymocytes expressing GFP-SLP-76 and GFP-MTS were purified from radiation chimeras, fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton, blocked (PBS plus 10% BSA), stained for CD3, washed, and mounted onto slides. Image contrast was enhanced and color channels were merged using IPLabs software.

Biochemistry

Thymocytes were stimulated with anti-CD3 (5 μg/ml), lysed, and resolved by SDS-PAGE or subjected to immunoprecipitation. For glycoprotein VI (GPVI) stimulation, purified platelets were resuspended in Walsh buffer (11) and stimulated with convulxin (CVX). Reactions were stopped with 2× lysis buffer. Western blotting used Abs to phosphotyrosine (4G10), phospho-phospholipase C (PLC)γ2 (Y11217), PLCγ2, pSLP-76 (Y1128), and actin.

Platelet degranulation and aggregation

Platelets were isolated as described (11). Degranulation was stimulated with CVX for 20 min at 37°C. CVX or collagen-stimulated aggregation was monitored in an aggregometer with stirring at 37°C by measuring changes in light transmission.

Results and Discussion

Impaired T cell development in MTS knockin mice

Because MTS partitions into lipid rafts and bypasses TCR signaling requirements for both SLP-76 and LAT in Jurkat cells (Ref. 12 and data not shown), we predicted that MTS would rescue SLP-76 deficiency in vivo. We generated genomic MTS knockin mice by fusing the LAT membrane targeting sequence to LCP2 exon 1. MTS/MTS mice were born at Mendelian ratios. Immunoblot analysis of MTS/+ thymocytes detected two species of SLP-76 protein: wild type (WT) (76 kDa) and MTS/MTS mice. The blot was probed for actin as a loading control. B, CD4 and CD8 expression of thymocytes from +/+ , MTS/+, and MTS/MTS mice. WT SLP-76 (76 kDa) were stained for CD3 (red).

FIGURE 1. Substantial blockade of T cell development in MTS knockin mice. A, SLP-76 expression in thymocytes from WT (+/+ ), MTS/+ , and MTS/MTS mice. The blot was probed for actin as a loading control. B, CD4 and CD8 expression of thymocytes from +/+ , MTS/+, and MTS/MTS mice. C, Membrane (Mem) and cytosolic (Cyt) cell fractions were prepared from resting thymocytes and immunoblotted for SLP-76 Ab. Blots were reprobed for β1 integrin (membrane marker) and ERK1,2 (cytosolic marker); n = 3. D, LAT-containing lipid rafts (lanes 3–5) were purified from resting thymocytes by solubilization in 1% Triton and density ultracentrifugation. SLP-76 partitioning into nonraft gradient fractions (lanes 9–13) was assessed by Western blotting. E, Resting thymocytes expressing GFP-tagged WT SLP-76 (left) or MTS (right) were stained for CD3 (red).
CD8 SP subpopulations. MTS heterozygous animals demonstrated normal thymocyte development and function compared with WT mice.

To investigate why MTS failed to support normal T cell development, we examined MTS localization. MTS was isolated in membrane preparations (Fig. 1C). But in contrast to our findings in Jurkat, MTS failed to partition with LAT in lipid raft preparations of resting thymocytes (Fig. 1D). By direct visualization, GFP-tagged MTS localized to internal membrane structures and, like WT SLP-76, failed to colocalize with either surface (Fig. 1E) or raft markers (not shown) in thymocytes. These findings indicate that peptide motifs may target cytosolic molecules differently in cell lines and primary cells and that the SLP-76 adapter function requires different localization during pre-TCR and mature TCR signaling.

MTS fails to couple the TCR to its signaling machinery in DP thymocytes

To understand why MTS failed to restore development of SP thymocytes, we investigated TCR signaling in thymocytes. Signals from the TCR that drive positive selection result in up-regulation of TCRβ and expression of the maturation markers CD69 and CD5 (1). Analysis of MTS DP thymocytes revealed fewer TCRβhigh and CD69high cells and reduced CD5 surface expression compared with WT. The few CD4 and CD8 SP cells that develop in MTS mice also express low levels of surface TCRβ (not shown). To directly test TCR signaling, WT or MTS thymocytes were stimulated by TCR crosslinking and analyzed biochemically. TCR-induced signals upstream of SLP-76, including phosphorylation of ZAP-70 and LAT, were unaffected or only mildly diminished in MTS thymocytes (not shown), yet tyrosine phosphorylation of the MTS itself was abolished (Fig. 2A). Strikingly, in stimulated MTS/WT thymocytes, only WT SLP-76 is phosphorylated despite equal expression of both MTS and WT proteins.

We have shown that deletion of SLP-76 in DP thymocytes uncouples TCR engagement from PLCγ1 phosphorylation and Ca2+ mobilization (13). MTS thymocytes demonstrated diminished TCR-induced PLCγ1 phosphorylation (not shown), and Ca2+ flux was dramatically reduced in DP MTS thymocytes (Fig. 2B). TCR signaling to the MAPK pathway was also defective in MTS thymocytes, as measured by phosphorylation of ERK1,2 (Fig. 2C). These data indicate that hypophosphorylated MTS fails to couple the TCR to activation of effector molecules in thymocytes.

LAT is required for MTS-supported thymic development

Our previous studies demonstrating that LAT is dispensable for MTS function in Jurkat cells (12) suggested that MTS might function independently of LAT in vivo. We thus examined the effect of LAT deletion in MTS mice, anticipating that if MTS could bypass the requirement for LAT, these mice would phenocopy LAT-sufficient MTS mice. LAT−/− mice demonstrate a complete block in thymocyte development at the DN3 stage (14). MTS expression failed to rescue this block at the DN stage (Fig. 3A).

Retroviral expression of cytosolic SLP-76 fails to rescue MTS T cell development

One possible explanation for the defect in the MTS thymus is that upon reaching the DP stage, thymocytes require a cytosolic pool of SLP-76. We coexpressed with MTS an SLP-76 mutant that cannot bind Gads (G2-SLP-76) and therefore cannot be recruited via LAT to the plasma membrane. MTS fetal liver cells were retrovirally transduced with GFP only (vector) or either GFP-tagged G2-SLP-76 or WT SLP-76 and were transplanted into lethally irradiated recipient mice. Following transplant, defective development was recapitulated in thymi of hosts reconstituted with MTS cells expressing vector, whereas expression of WT SLP-76 rescued the defect (Fig. 3B). In
contrast, expression of G2-SLP-76 failed to restore MTS thymocyte development beyond the DP stage. Thus, defective T cell development in MTS mice does not result from the absence of a cytosolic pool of SLP-76.

**Intact phosphorylation and function of MTS in platelets**

The failure of MTS to support TCR signaling in thymocytes raised the question of whether MTS can function in any primary cell type. Therefore, we investigated MTS platelet signaling downstream of the GPVI collagen receptor. Like the TCR, GPVI signals via an ITAM-based PTK pathway mediated by Src and Syk kinases, LAT, SLP-76, and PLCγ2 (15). Consistent with previous findings, SLP-76−/− platelets failed to up-regulate surface expression of P-selectin in response to CVX (9), whereas WT cells demonstrated a dose-dependent response. GPVI-induced P-selectin expression was completely restored in MTS platelets (Fig. 4A). MTS and WT platelets mounted comparable aggregation responses to CVX or collagen stimulation (Fig. 4B).

We next asked whether MTS is phosphorylated upon GPVI stimulation. Treatment of platelets with CVX resulted in inducible phosphorylation of both SLP-76 and MTS, as measured by 4G10 or pY128 SLP-76 immunoblotting (Fig. 4C). Furthermore, phosphorylation of PLCγ2 was induced to equal levels in MTS and WT platelets (Fig. 4D), indicating that SLP-76-dependent function downstream of the GPVI is fully restored by MTS.

We were surprised that MTS failed to support thymic development but rescued TCR signaling in SLP-76−/− and LAT−/− Jurkat T cells (12). We considered that MTS may be unable to function in vivo. However, the near complete rescue of pre-TCR signaling and the restoration of signaling via GPVI indicated that this was not the case. Given the robust MTS GPVI response in platelets, we next speculated that augmented signaling by MTS could drive thymic negative selection. It was also possible that the MTS transmembrane moiety disrupted the function of endogenous LAT. However, both the normal development of MTS heterozygotes and the developmental rescue of MTS by retroviral expression of WT SLP-76 argue that the MTS phenotype represents a loss rather than a gain of function. Our third model for failed thymocyte development was that certain SLP-76-dependent signaling may be restricted to the cytosol and cannot be organized by a membrane-bound protein. Failed reconstitution with G2-SLP-76 demonstrated that this cannot explain the MTS defect.

One hint to the failure of MTS to support thymic development is defective MTS phosphorylation in response to TCR crosslinking. Tyrosine phosphorylation of SLP-76 is required for activation of effector molecules (16). Following TCR stimulation, ZAP-70 relocates to mobile microclusters at the cell surface (17, 18), and if MTS cannot access the surface, its phosphorylation may be diminished. However, phosphorylation of the G2-SLP-76 mutant, despite its failure to relocate to the cell surface, suggests that ZAP-70 may be active in multiple locations within the cell (8). It is possible that DP thymocytes restrict productive TCR signaling to the cell surface, whereas DN thymocytes and platelets permit the assembly of multimolecular signaling complexes at intracellular sites. This is consistent with described differences in the requirements for these receptors to function. Early studies (19) documented that the pre-TCR could transduce signals in a ligand-independent fashion and perhaps without trafficking to the cell surface. More recent studies (20) indicate that the pre-TCR signals within preformed membrane complexes, whereas the mature TCR must be recruited into these membrane regions. Our work extends these studies by showing that SLP-76, a key integrator of both pre-TCR and TCR signals, supports signaling from these two receptors at different locations within the cell.

Comparing TCR and GPVI signaling in MTS mice provides additional insight contrasting the requirements for adapter localization in two primary cell systems, both of which use ITAMs. Intact MTS function in platelets may result from differential localization in platelets vs thymocytes. Additionally, platelets and early thymocytes rely on the Syk PTK for signal generation, whereas the mature TCR relies instead on ZAP-70. Others have shown that the rules for Syk vs ZAP-70 activation differ considerably (21). Concordantly, we found that MTS is inducibly phosphorylated on tyrosines following GPVI engagement on platelets but not in response to TCR ligation in thymocytes. Thus, it is possible that one other difference between Syk and ZAP-70 is where in the cell these two PTKs can act. In preliminary experiments, overexpression of Syk in MTS fetal liver cells resulted in partial restoration of SP thymocyte development and the appearance of peripheral T cells.

![FIGURE 4. MTS functions as efficiently as WT SLP-76 during GPVI signaling in platelets. A. +/+ or MTS/MTS platelets were stimulated with CVX in the presence of anti-P-selectin Ab. Mean percentages (±SEM) of P-selectin+ platelets are shown and are representative of WT and MTS mice (n = 4) and SLP-76−/− (n = 2) mice. Unstim, Unstimulated. B. Platelets were stimulated with 1 nM CVX or 10 μg/ml collagen. Aggregation was measured as a percentage change in OD. The time at which the agonist was added is indicated by an arrowhead; n = 3. C. +/+ or MTS/MTS platelets were stimulated with CVX and lysed by direct pY128 immunoblotting and by immunoprecipitation (IP) with SLP-76 Ab followed by 4G10 immunoblotting and by pY128 SLP-76 immunoblotting. Blots were reprobed for total SLP-76; Lysate, lysed in 2x lysis buffer. MTS phosphorylation was measured by immunoprecipitation (IP) with SLP-76 Ab followed by 4G10 immunoblotting and by direct pY128 immunoblotting. Blots were reprobed for total SLP-76; n = 3. D. PLCγ2 phosphorylation was detected by immunoblotting and reprobed for total PLCγ2; n = 3.]
Analysis of MTS mice has offered new understanding of how adapter protein localization regulates immunoreceptor signals. First, our data caution that targeting protein subcellular localization depends on the cell type. Our data also reinforce the notion that requirements for signaling via the pre-TCR and the mature TCR overlap but are not identical. Receptors in different lineages, despite shared signaling components, do not possess identical requirements for the subcellular localization of at least some critical elements. Assumptions of cell types based on discoveries in other lineages should be made carefully despite similar paradigms for signaling. Finally, although T cell development is impaired in the MTS mice, some SP thymocytes and mature T cells arise. In preliminary analysis, rather than remaining inert due to impaired TCR signaling, these peripheral T cells are activated and skew toward production of inflammatory cytokines. How the partial rescue of TCR signaling by MTS may predispose peripheral T cells to specific effector fates is currently under investigation.

Acknowledgments
We thank the Koretzky laboratory for discussion and Taku Kambayashi for critical review of this manuscript.

Disclosures
The authors have no financial conflict of interest.

References