Functional Complementation of BLNK by SLP-76 and LAT Linker Proteins*

Received for publication, May 24, 2000
Published, JBC Papers in Press, August 8, 2000, DOI 10.1074/jbc.M004467200

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Recent studies have demonstrated a requirement for the SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) and LAT (linker for activation of T cells) adapter/linker proteins in T cell antigen receptor activation and T cell development as well as the BLNK (B cell linker) linker protein in B cell antigen receptor (BCR) signal transduction and B cell development. Whereas the SLP-76 and LAT adaptor proteins are expressed in T, natural killer, and myeloid cells and platelets, BLNK is preferentially expressed in B cells and monocytes. Although BLNK is structurally homologous to SLP-76, BLNK interacts with a variety of downstream signalling proteins that interact directly with both SLP-76 and LAT. Here, we demonstrate that neither SLP-76 nor LAT alone is sufficient to restore the signaling deficits observed in BLNK-deficient B cells. Conversely, the coexpression of SLP-76 and LAT together restored BCR-inducible calcium responses as well as activation of all three families of mitogen-activated protein kinases. Together, these data suggest functional complementation of SLP-76 and LAT in T cell antigen receptor function with BLNK in BCR function.

Engagement of T and B cell receptors results in the sequential activation of three distinct classes of cytoplasmic protein-tyrosine kinases (PTKs) (reviewed in Refs. 1–3). The Src kinases (Lck and Fyn in T cells and Lyn, Blk, and Fyn in B cells) phosphorylate two tyrosine residues within each of the immunoreceptor tyrosine-based activation motifs encoded within the signaling polypeptides of the T and B cell antigen receptors. Phosphorylation of both tyrosines within the immunoreceptor tyrosine-based activation motif provides docking sites for the binding of the Syk family of PTKs (Syk and ZAP-70) with the receptor. The localization of the Syk PTKs to the receptor and Src PTKs results in enzymatic activation of ZAP-70 and Syk, which is required for efficient generation of downstream second messengers. In addition to the Src and Syk families of PTKs, the Tec family of PTKs (Rlk and Itk in T cells and Btk in B cells) is also activated following receptor engagement (reviewed in Refs. 4 and 5). This third family of PTKs plays a requisite role in regulating increases in free cytoplasmic calcium levels ([Ca2+]i) following receptor engagement.

The efficient and coordinated generation of a number of second messengers is required for normal lymphocyte function (reviewed in Ref. 6). These second messengers include increases in [Ca2+]i, and the generation of GTP-bound forms of Ras and Rho GTPases. Increases in [Ca2+]i, require the enzymatic activation of the γ1 and γ2 isoforms of phospholipase C (PLCγ1 and PLCγ2), which is mediated, in part, by the tyrosine phosphorylation of these enzymes by antigen receptor-activated PTKs (7–9). The hydrolysis of phosphatidylinositol bisphosphate to inositol trisphosphate and diacylglycerol induces increases in [Ca2+]i, and activates protein kinase C isoenzymes, respectively. These calcium transients activate many Ca2+-dependent enzymes, including the calcineurin serine/threonine phosphatase, to control the subcellular localization of the NF-AT (nuclear factor of activated T cells) transcription factor.

The generation of GTP-bound forms of Ras and the Rho family of GTPases is mediated by a variety of exchange factors (reviewed in Ref. 10). Recruitment of Sos, a guanine nucleotide exchange factor, to the plasma membrane through the Grb2 adaptor molecule results in the generation of Ras-GTP and the activation of the Raf-MEK-ERK pathway. In addition, diacylglycerol can bind and activate Ras-GTP, which, in turn, may couple changes in diacylglycerol and possibly [Ca2+]i, to Ras activation (11). The Rho family of GTPases is regulated, in part, by the Vav guanine nucleotide exchange factor (12). Activation of Vav regulates a number of downstream signaling pathways, including the activity of all three MAPK families, [Ca2+]i, and activation of protein kinase Cδ, the latter of which is also required for the activation of NF-κB (13–17).

Recent studies have underscored the importance of hematopoietic cell-specific linker or adaptor proteins in bridging the antigen receptor-associated cytoplasmic PTKs with the activation of these downstream effector proteins (reviewed in Refs. 10 and 18–21). In T cells, the transmembrane LAT and cytosolic SLP-76 proteins are preferentially phosphorylated by the activated ZAP-70 and Syk PTKs after T cell antigen receptor (TCR) activation (22–24). Phosphorylated LAT provides docking sites for SH2 domain-containing molecules, including PLCγ1, phosphatidylinositol 3-kinase, and the Grb2 and Gads (also known as GrpL, MonA, and Grf40) adaptor proteins (24–27). The interaction of Grb2 with Sos permits the localization of the Grb2-Sos complex to the plasma membrane, which is thought to
facilitate exchange of GDP for GTP on Ras (28, 29). Cells lacking LAT are unable to mobilize calcium and have reduced ERK2 activation upon TCR engagement (30). Phosphorylation of SLP-76 similarly facilitates the assembly of macromolecular signaling complexes (23, 26, 31–34). Phosphorylation of distinct tyrosines within SLP-76 facilitates the binding of both Vav and Nck adaptor proteins (34, 35). The assembly of Vav-SLP-76-Nck complexes co-localizes the Vav guanine nucleotide exchange factor for GTPases with some of its targets, including the Rho GTPase-dependent Pak serine/threonine kinase. SLP-76 also binds Gads, which facilitates the binding of SLP-76-Gads complexes to tyrosine-phosphorylated LAT (25–27). The assembly of these macromolecular complexes may, in turn, provide mechanisms for cross-talk among different signaling pathways for signal integration. Consistent with this model, SLP-76-deficient Jurkat T cells also demonstrate defects in \([\text{Ca}^{2+}]_i\), and ERK activation following TCR engagement (36).

As neither SLP-76 nor LAT is expressed in B cells, recent studies have demonstrated that the BLNK protein (also known as SLP-65 and BASH) serves to bridge the receptor-activated PTKs with downstream effector enzymes in B cells (reviewed in Refs. 37–40). Phosphorylation of BLNK by Syk provides docking sites for a number of downstream effector proteins, including Nck, Vav, PLC γ, and Btk, and augments its interaction with Grb2 (38, 40, 41). Similar to the phenotypes of LAT- and SLP-76-deficient Jurkat T cells, BLNK-deficient DT40 B cells do not increase \([\text{Ca}^{2+}]_i\), and do not efficiently activate the three families of MAPKs after B cell antigen receptor (BCR) cross-linking (42). Although BLNK is structurally homologous to SLP-76 (33% amino acid homology), BLNK also binds downstream effectors such as PLC γ and Grb2. In T cells, these latter effectors interact directly with LAT. Hence, BLNK is capable of interacting with downstream effectors that bind both SLP-76 and LAT. To test the functional homologies between the T and B cell linker proteins, we examined the ability of the T cell SLP-76 and LAT linker molecules to complement the function of the B cell BLNK protein.

EXPERIMENTAL PROCEDURES

Cells and cDNAs—Conditions for growth of BLNK−/− DT40 B cells and JcAM2 and J14 T cells have been previously described (30, 36, 42). The cDNAs for murine SLP-76, murine LAT, and chicken BLNK were generated by polymerase chain reaction, and the sequences were confirmed by DNA dideoxy sequencing (24, 42, 43). Both SLP-76 and LAT represent functional homologues of BLNK, cDNAs were generous gifts from Drs. Ed Clark (University of Washington) and Aubrey Morrison (Washington University, St. Louis, MO), respectively.

Antibodies—The antibodies used and their sources included the following: M4, an anti-clonotypic chicken IgM monoclonal antibody (mAb) for DT40 B cells (42); anti-Myc mAb (Covance); anti-LAT Ab (Upstate Biotechnology, Inc.); anti-SLP-76 Ab (22); anti-phospho-ERK2 Ab (Promega); anti-phospho-JNK Ab (Promega); anti-phospho-p38 Ab (Santa Cruz Biotechnology); anti-ERK2 Ab (Santa Cruz Biotechnology); anti-JNK Ab (Santa Cruz Biotechnology); anti-p38 Ab (Santa Cruz Biotechnology); anti-FLAG mAb M5 (Eastman Kodak Co.); anti-actin mAb (Sigma); and anti-phosphotyrosine mAbs PY20 and 4G10 (Santa Cruz Biotechnology and Upstate Biotechnology, Inc., respectively).

Generation of Cells—SLP-76, LAT, and BLNK were subcloned into the pApuro vector and linearized prior to electroporation. Cells were electroporated at 300 V, 960 microfarads, and resistance setting (R9) with a BTX Electroporator. Forty-eight hours following electroporation, cells were selected in puromycin (0.5 μg/mL)-containing medium.

Conditions for BCR Activation—Cells were suspended in phosphate-buffered saline at 106 cells/ml and rested at 37 °C for 15 min prior to stimulation. Cells were stimulated with 2 μg/ml anti-BCR mAb M4 for the indicated time points; quickly sedimented by centrifugation; and lysed at 106 cells/ml in 10 mm Tris, pH 8.0, 150 mm NaCl, and 1% Nonidet P-40 with protease and phosphatase inhibitors as described previously (38). Cell debris was sedimented by centrifugation at 14,000 × g for 10 min at 4 °C. Clarified supernatants were readied for additional analysis.

For NF-AT assays, 106 cells were transfected with 40 μg of the desired cDNA and 30 μg of an NF-AT-responsive luciferase reporter construct. Forty-eight hours following transfection, the cells were incubated with medium alone, medium containing 2 μg/mL mAb M4, or medium containing phorbol 12-myristate 13-acetate and ionomycin as described previously (38).

Detection of \([\text{Ca}^{2+}]_i\)—Cells were loaded with Fura-2 (Molecular Dynamics, Inc.), and fluorescence was monitored using a Hitachi F2000 fluorescence spectrophotometer at wavelengths of 340 and 540 nm according to the manufacturer’s recommendations. Cells were rested for 15 min at 37 °C prior to stimulation with soluble mAb M4 (2 μg/ml). Maximal fluorescence was determined following lysis of cells with 1% Triton X-100; minimal fluorescence was measured following chelation of \([\text{Ca}^{2+}]_i\), with 10 mM EGTA.

RESULTS

Generation of BLNK-deficient Cells That Express SLP-76, LAT, or Both SLP-76 and LAT—To determine whether SLP-76 and LAT represent functional homologues of BLNK, cDNAs encoding either SLP-76 or LAT were expressed in BLNK−/− DT40 B cells as described under “Experimental Procedures.”
Immunoblot analysis of representative clones of SLP-76 and LAT revealed a spectrum of expression levels. Clones expressing comparable or greater levels of each of these linker proteins compared with the endogenous proteins expressed in Jurkat T cells were selected for further analysis (Fig. 1, A and B). To examine the functions of both SLP-76 and LAT relative to BLNK, we also generated stable clones that expressed both SLP-76 and LAT in BLNK−/− DT40 B cells. Immunoblot analysis of the representative clones revealed a ratio of expression of SLP-76 and LAT in the BLNK-deficient cells that was comparable to that in Jurkat T cells (Fig. 1C). Quantitation of actin was performed to control for equalization of protein loading in each lane (Fig. 1, A–C, lower panels). Each of the clones selected for analysis expressed comparable levels of surface BCR expression as judged by fluorescence-activated cell sorter staining (data not shown).

Expression of neither SLP-76 nor LAT Alone Is Sufficient to Restore BLNK Function—Since BLNK is required for the BCR-induced increase in [Ca2+]i, following BCR cross-linking (Fig. 2, A and B), we analyzed the ability of the single transfectants expressing either SLP-76 or LAT to increase [Ca2+]i, following BCR engagement. Expression of SLP-76 alone in BLNK−/− cells failed to restore BCR-induced calcium responses (Fig. 2, C and D). Analysis of additional clones that expressed up to 4-fold greater levels of SLP-76 as compared with clone 2A2 failed to produce any additional calcium responses (data not shown). Most clones expressing SLP-76 alone demonstrated undetectable calcium transients that were comparable to those of the 2A2 clone, although a small calcium flux (maximal [Ca2+]i increase of 20 nM) was detected in clone 1C4 following BCR cross-linking. Similarly, no tyrosine phosphorylation of PLCγ2 was detected in these clones following BCR cross-linking (data not shown). Conversely, BLNK−/− cells expressing LAT gave rise to a BCR-inducible calcium response that was approximately one-fourth of the level in cells expressing wild-type BLNK in all clones analyzed (Fig. 2, E and F; and data not shown). Consistent with this attenuated calcium response, cells expressing LAT alone demonstrated tyrosine phosphorylation of PLCγ2 that was significantly diminished compared with cells expressing wild-type BLNK (data not shown).

Cells expressing either SLP-76 or LAT alone also demonstrated significantly diminished BCR-induced NF-AT-regu-
lated responses (Fig. 2). The small degree of receptor-induced response shown for clones 4A10 (expressing SLP-76) and 7G11 (expressing LAT) represented the maximal response observed among the panel of clones analyzed. Experiments using transient transfections of either SLP-76 or LAT cDNAs into $\text{BLNK}^{-/-}$ B cells also failed to result in any receptor-inducible activation of the NF-AT-responsive reporter gene (data not shown). Hence, neither SLP-76 nor LAT alone is sufficient to reconstitute the BLNK-mediated calcium or calcium-dependent responses.

As BLNK is also required for the efficient activation of all three families of MAPKs, ERK, JNK, and p38 (42), we next analyzed the ability of SLP-76 or LAT to regulate these three MAPKs. Whereas $\text{BLNK}^{-/-}$ cells demonstrated an attenuated level and kinetics of ERK2 activation compared with cells expressing wild-type BLNK, cells expressing either SLP-76 or LAT alone did not reconstitute ERK2 activation to levels found in cells expressing wild-type BLNK irrespective of the duration of stimulation (Fig. 3A). Analysis of other LAT- or SLP-76-expressing clones revealed a similar defect (data not shown). Hence, neither SLP-76 nor LAT alone is sufficient to reconstitute the BCR-mediated activation of any of the three families of MAPKs.

Since tyrosine phosphorylation of SLP-76 and LAT is required for their ability to activate downstream signaling functions (22–44), we analyzed the phosphorylation status of SLP-76 or LAT from resting or BCR-activated $\text{BLNK}^{-/-}$ DT40 B cells expressing wild-type BLNK, SLP-76, or LAT. Forty-eight hours following transfection, the cells were cross-linked with an anti-BCR mAb (M4) for the indicated time periods and lysed, and p38 was immunoprecipitated with and anti-FLAG mAb. Anti-p38 immunoprecipitates were analyzed by immunoblot analysis with anti-phospho-p38 (upper panel) or anti-total p38 (lower panel) antiserum.

![Fig. 3. Activation of MAPKs in cells expressing either SLP-76 or LAT.](image)

**Fig. 3. Activation of MAPKs in cells expressing either SLP-76 or LAT.** A, activation of ERK2. $2 \times 10^6 \text{BLNK}^{-/-}$ DT40 B cells or $\text{BLNK}^{-/-}$ DT40 B cells expressing wild-type BLNK, SLP-76, or LAT were cross-linked with an anti-BCR mAb (M4) for the indicated time periods. Cells were lysed as described under “Experimental Procedures” and analyzed by immunoblot analysis for phospho-ERK2 (pERK2; upper panel) and total ERK2 (lower panel). The slower migrating band present in all lanes represents a nonspecific band immunoreactive to the secondary Ab used (data not shown). B, activation of JNK. Cells were analyzed for phospho-JNK (pJNK; upper panel) and total JNK (lower panel) as described for A. C, activation of p38. FLAG-tagged p38 cDNA was electroporated into $\text{BLNK}^{-/-}$ DT40 B cells or $\text{BLNK}^{-/-}$ DT40 B cells expressing wild-type BLNK, SLP-76, or LAT. Forty-eight hours following transfection, the cells were cross-linked with an anti-BCR mAb (M4) for the indicated time periods and lysed, and p38 was immunoprecipitated with and anti-FLAG mAb. Anti-p38 immunoprecipitates were analyzed by immunoblot analysis with anti-phospho-p38 (upper panel) or anti-total p38 (lower panel) antiserum.

![Fig. 4. Tyrosine phosphorylation of SLP-76 and LAT.](image)

**Fig. 4. Tyrosine phosphorylation of SLP-76 and LAT.** SLP-76 or LAT was immunoprecipitated from resting or BCR-activated $\text{BLNK}^{-/-}$ DT40 B cells expressing SLP-76 (A) or LAT (B) alone. Immunoprecipitates were analyzed by immunoblot analysis with anti-phospho-Tyr mAbs (anti-pTyr; upper panels) or anti-SLP-76 or anti-LAT antibodies (lower panels).
their phosphorylation in the BLNK−/− DT40 cells.

SLP-76 and LAT Synergize to Functionally Compensate for BLNK—Since neither SLP-76 nor LAT alone can restore BLNK-mediated activation of downstream signaling events, we tested whether the combination of SLP-76 and LAT could functionally complement the absence of BLNK. Cells expressing both SLP-76 and LAT demonstrated BCR-induced and sustained calcium fluxes that were comparable to or greater than those observed in wild-type cells (Fig. 5A and data not shown). In addition, the tyrosine phosphorylation of PLC-γ2 was restored to levels detected in cells expressing wild-type BLNK (data not shown). Consistent with the restoration of receptor-induced increases in [Ca2+]i, the NF-AT-regulated response was also restored to levels comparable to or greater than those observed in wild-type cells (Fig. 5B).

The coexpression of SLP-76 and LAT also restored the ability of BLNK−/− cells to activate ERK, JNK, and p38 following BCR cross-linking (Fig. 6, A–C). In fact, the level of activated ERK, JNK, and p38 observed in cells expressing both SLP-76 and LAT was consistently higher compared with cells expressing wild-type BLNK (Fig. 6B). This increased activation may simply reflect a greater molar ratio of expressed SLP-76 and LAT compared with endogenous BLNK or may suggest that the down-regulation of antigen receptor function by SLP-76 and LAT differs from that regulated by BLNK.

Finally, we analyzed the phosphorylation status of coexpressed SLP-76 and LAT. Similar to cells expressing SLP-76 or LAT alone, SLP-76 and LAT were inducibly phosphorylated in cells expressing both SLP-76 and LAT (Fig. 7, A and B). In addition, whereas tyrosine-phosphorylated LAT co-immunoprecipitated with SLP-76 in Jurkat T cells following TCR cross-linking (Fig. 7A, lane 6), the slower migrating epitope-tagged LAT was also phosphorylated and co-immunoprecipitated with SLP-76 following BCR cross-linking (Fig. 7A, lanes 8 and 10; and data not shown). Together, these data suggest that SLP-76 and LAT together are required to complement BLNK function.

DISCUSSION

Our studies here demonstrate a requirement for both SLP-76 and LAT to complement the absence of BLNK. BLNK−/− cells expressing LAT alone were unable to efficiently increase [Ca2+]i or activate the three MAPK families following BCR cross-linking. However, a small increase in free [Ca2+]i was consistently observed in these LAT-expressing BLNK−/− cells, which is consistent with the attenuated, but measurable, calcium responses observed in SLP-76-deficient Jurkat T cells and slp-76−/− mast cells following TCR and Fcε receptor type I engagement, respectively (36, 45). Conversely, SLP-76-expressing BLNK−/− cells demonstrated a minimal (if any) increase in [Ca2+]i, following receptor engagement, which is consistent with the decreased to absent calcium response observed in LAT-deficient Jurkat T cells and lat−/− mast cells (30). In contrast, the coexpression of both SLP-76 and LAT to levels similar to the ratio expressed in Jurkat T cells was sufficient to restore calcium and MAPK responses to the levels detected in cells expressing wild-type BLNK. Since tyrosine-phosphory-
lated BLNK interacts with downstream effector proteins (including Vav, Nck, PLC\(_\gamma\), Grb2, and Btk) that directly interact with both SLP-76 (Vav and Nck) and LAT (PLC\(_\gamma\), Gads, and Grb2), the studies presented here support the notion that BLNK not only serves as a functional and structural homologue of SLP-76 in B cells, but also encompasses the downstream signaling functions of LAT.

In addition to SLP-76 and LAT, Gads, a member of the Grb2 family of adaptor proteins, constitutively associates with SLP-76 and can also bind tyrosine-phosphorylated LAT (25–27). Studies in T cells and in vitro mapping analysis have suggested a role for Gads in linking the LAT and SLP-76 signaling complexes (25). In our studies here, we were able to co-immunoprecipitate tyrosine-phosphorylated LAT with SLP-76 following BCR cross-linking in cells expressing both SLP-76 and LAT. Since Gads protein and mRNA are not detected in DT40 cells (data not shown), the presence of Gads is not absolutely required in these clones. However, the additional expression of Gads in BLNK\(^{-/-}\) DT40 cells that coexpress SLP-76 and LAT is able to further augment NF-AT-regulated promoter responses (data not shown). These data are consistent with the notion that Gads can promote the efficient assembly of LAT-SLP-76 complexes following receptor activation (25). In addition, whereas lat\(^{-/-}\) and slp-76\(^{-/-}\) mice accumulate CD4\(^+\)CD8\(^-\) double-negative thymocytes and do not develop CD4\(^+\)CD8\(^+\) or single-positive (CD4\(^+\) or CD8\(^+\)) thymocytes, gads\(^{-/-}\) mice develop some CD4\(^+\)CD8\(^-\) and CD4\(^-\)/CD8\(^+\) thymocytes, although the presence of Gads is required for efficient TCR function.\(^2\) Hence, the pre-TCR, which can utilize either ZAP-70 or Syk to facilitate the transition of double-negative to double-positive T cells, can activate some downstream signaling functions to generate double-negative T cells in the absence of Gads (47).\(^2\) The ability of the cells described here that express both SLP-76 and LAT to compensate for BLNK in the absence of Gads might also reflect the expression of Gads-related molecules, such as Grap and Grb2, in this system (46, 48).

\(^2\) C. Pham, Y. Iizuka, J. Yoder, S. Liu, J. McGlade, and A. M. Cheng, submitted for publication.

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**FIG. 6.** Restoration of MAPK activation in cells expressing both SLP-76 and LAT. A, activation of ERK2. 2 \times 10^5 BLNK\(^{-/-}\) DT40 B cells or BLNK\(^{-/-}\) DT40 B cells expressing wild-type BLNK or SLP-76 and LAT were cross-linked with an anti-BCR mAb (M4) for the indicated time periods. Cells were lysed as described under “Experimental Procedures” and analyzed by immunoblot analysis for phospho-ERK2 (pERK2; upper panel) and total ERK2 (lower panel). B, activation of JNK. Cells were analyzed for phospho-JNK (pJNK; upper panel) and total JNK (lower panel) as described for A. C, activation of p38. FLAG-tagged p38 cDNA was electroporated into BLNK\(^{-/-}\) DT40 B cells or BLNK\(^{-/-}\) DT40 B cells expressing wild-type BLNK or SLP-76 and LAT (clone 7B9). The analysis of these clones followed the protocol described for Fig. 3C.

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**FIG. 7.** Tyrosine phosphorylation of SLP-76 and LAT in cells expressing both SLP-76 and LAT. A, tyrosine phosphorylation of SLP-76 and its associated proteins. Resting or activated cells were immunoprecipitated with an anti-SLP-76 mAb (H3) and analyzed by immunoblot analysis with anti-phospho-Tyr mAbs (anti-pTyr; upper panel) or anti-SLP-76 mAb (lower panel). The migration positions of tyrosine-phosphorylated LAT and Myc-tagged LAT proteins are denoted. B, tyrosine phosphorylation of LAT. Resting or activated cells were immunoprecipitated with an anti-LAT Ab and analyzed by immunoblot analysis with anti-phospho-Tyr mAbs (upper panel) or anti-LAT antiserum (lower panel).

Finally, although the coexpression of SLP-76 and LAT was able to compensate for the absence of BLNK in the DT40 B cell system, the expression of BLNK in LAT-deficient (JCaM2) or SLP-76-deficient (J14) Jurkat T cells failed to reconstitute TCR-mediated calcium and MAPK responses (data not shown). The failure of this reconstitution was, in part, due to the inability of BLNK to be efficiently phosphorylated following TCR...
cross-linking (data not shown). Furthermore, expression of a membrane-localized form of BLNK was also unable to be phosphorylated or to restore TCR-mediated functions following TCR engagement (data not shown). Hence, it is likely that additional B cell-specific molecules are required to properly localize BLNK to the receptor-activated PTKs for efficient phosphorylation. Preliminary studies suggest that the SH2 domain of BLNK may mediate this localization,3 and additional studies are ongoing to identify the molecules that mediate this interaction.

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