if no clearance occurred (i.e., 5% of estimated blood volume of the primary recipient animal, usually ~2.5 ml based on weight, divided by the number of "direct transfer" transplant recipients). Data were analyzed by Kruskal-Wallis one-way nonparametric analysis of variance and Mann-Whitney U tests, and differences between the "direct transfer" group and the "prebleed," "30 s," and "6 min" groups were statistically significant ($P < 0.05$).

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T Cell Responses Modulated Through Interaction Between CD8αα and the Nonclassical MHC Class I Molecule, TL

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The thymus leukemia antigen (TL) is a nonclassical class I molecule, expressed abundantly on intestinal epithelial cells. We show that, in contrast to other major histocompatibility complex (MHC) class I molecules that bind CD8αβ, TL preferentially binds the homotypic form of CD8α (CD8αα). Thus, TL tetramers react specifically to CD8αα-expressing cells, including most intestinal intraepithelial lymphocytes. Compared with CD8αβ, which recognizes the same MHC as the T cell receptor (TCR) and thus acts as a TCR coreceptor, high-affinity binding of CD8αα to TL modifies responses mediated by TCR recognition of antigen presented by distinct MHC molecules. These findings define a novel mechanism of lymphocyte regulation through CD8αα and MHC class I.

Several nonclassical class I molecules are encoded in the *T* region of the mouse MHC. These proteins are antigens and are named after the thymus leukemia antigen (TL) encoded by the *T3/T18* gene pair (*1*). It is striking that TL displays nearly exclusive expression on epithelial cells of the small intestine (*2*). The expression by intestinal epithelial cells has led to the hypothesis that TL could be recognized by TCRs expressed on intraepithelial lymphocytes (IELs) (*3*). IELs are an enigmatic subset of predominantly $CD8^+$ T lymphocytes, which reside among epithelial cells. The unique location of these cells suggests that they may function in host defense, surveillance for damaged epithelium, or immune regulation.

To identify T cells that might interact with TL, we generated TL tetramers using a baculovirus expression system (*4*). As shown in Fig. 1A, TL tetramers stained the majority of IELs, but not splenocytes, and only a small minority of thymocytes. Tetramer binding was independent of TCR specificity, and it bound TCRαβ and TCRγδ cells equally well (*5*). Thus, expression of the TL receptor by IELs was distinct from the TCR. The staining of IELs with insect cell–derived TL indicates that tetramer binding was also independent of peptide loading to TL, consistent with previous evidence that TL does not bind peptides (*6*).

The α3 domain of TL conserves the CD8α-binding motif defined for class I molecules (*7*). In light of the specific binding to IELs, which express the homodimeric form of CD8, CD8αα, we reasoned that TL tetramers might bind this invariant molecule. Consistent with this, IELs from $CD8\alpha^{-/-}$ mice showed an almost complete absence of staining with the TL tetramer, whereas no reduction was observed on IELs from $CD8\beta^{-/-}$ mice (Fig. 1A). Similarly, thymocytes from $CD8\beta^{-/-}$ mice, which in the absence of CD8β express CD8αα homodimers, showed elevated TL tetramer binding, as did the few remaining $CD8^+$ splenocytes (Fig. 1A). Collectively, these data suggest that CD8αα, but not CD8αβ, forms a specific receptor for the TL tetramer.

The $CD8^-CD3^-$, TCR-deficient BW5147 thymoma did not stain with TL tetramers unless first transfected with CD8α (Fig. 1B), providing further evidence that tetramer binding is not TCR-dependent or IEL-specific. Similarly, transfectants of the T cell hybridoma BI-141 expressing CD8α alone reacted with the TL tetramer, whereas cells expressing CD4 did not (Fig. 1C). An antibody against TL (Fig. 1B), as well as an antibody against CD8α (Fig. 1C), could inhibit tetramer binding. Unlike $CD8\alpha\beta^+$ splenocytes, the TL tetramer bound the $CD8\alpha\beta^+$ transfectants too (Fig. 1C). However, multistep reciprocal immunoprecipitations (*8*) revealed that large numbers of CD8αα molecules were coexpressed with CD8αβ (Fig. 1D) and suggest that CD8αα might also be coexpressed on the TL tetramer binding $CD8\alpha\beta^+$ IELs (*5*).

To confirm the specific interaction of TL with CD8αα, direct binding studies were performed by surface plasmon resonance (*9*). TL monomer binding to CD8αα immobilized on a biosensor chip (*10*) exhibited fast association and disassociation rates, with an equilibrium-binding constant (K_D) of 12 μM (Fig. 2A). By contrast, saturation of TL binding with CD8αβ could not be reached at the highest concentration of TL (Fig. 2B). Consequently, an accurate K_D value could not be determined, although Scatchard analysis indicated a value of at least 90 μM (*11*). The class I molecule K^b did not show such a propensity and, in agreement with previous results, (*10*) bound with comparable affinity to CD8αα and CD8αβ (Fig. 2). These data directly demonstrated a preferential and relatively high affinity binding of TL to CD8αα.

We used a CD8α-deficient T cell hybridoma specific for SIINFEKL/H-$2K^b$ (*12*), and a CD8α-transfected variant, to examine the effects of CD8αα-TL binding on TCR-mediated responses (*13*). OVA peptide-loaded RMA-H (TL^-) thymoma cells, or TL-transfected variants, were used to stimulate the T cells. Upon antigen activation by CD8αα-expressing target cells, TL^+ stimulator cells showed a significant-

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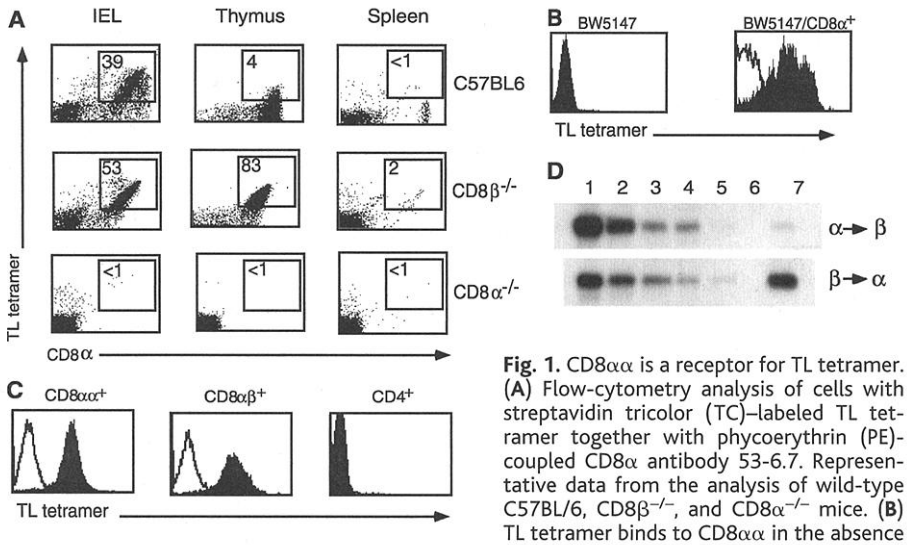

Fig. 1. CD8αα is a receptor for TL tetramer. (**A**) Flow-cytometry analysis of cells with streptavidin tricolor (TC)–labeled TL tetramer together with phycoerythrin (PE)-coupled CD8α antibody 53-6.7. Representative data from the analysis of wild-type C57BL/6, CD8β$^{-/-}$, and CD8α$^{-/-}$ mice. (**B**) TL tetramer binds to CD8αα in the absence of a TCR. The solid histograms show the results from flow-cytometry analysis of TC-labeled TL tetramer staining of TCR-deficient parent cell line BW 5147 (left) and the CD8α-transfected variant, BW5147/CD8α$^+$ (right). Blocking of the TL tetramer staining with an TL-specific mAb is shown (open histogram). (**C**) TL tetramer binding (solid) can be blocked with CD8α mAb (open), as described (*4*). BI-141 T cell hybridoma cells transfected with CD8α, both CD8α and CD8β, or CD4 (*28*) were analyzed. (**D**) Tetramer binding CD8αβ$^+$ cells coexpress CD8αα. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of CD8αβ transfectants of BI-141 that were surface-labeled with ^{125}I. Cells were sequentially immunoprecipitated (*8*) with CD8α mAb (top row, lanes 1 to 5), followed by CD8β immunoprecipitation (lane 7). Lane 6 is empty. Sequential CD8β immunoprecipitations (bottom row, lanes 1 to 5) were followed CD8α immunoprecipitation (lane 7). Representative data in (A) to (D) are shown from one of several experiments in every case.

ly enhanced interleukin 2 (IL-2) release (Fig. 3A), which could be blocked by TL-specific antibody (Fig. 3A). The TL-mediated increase in cytokine release was further confirmed using IELs from TCR transgenic mice (*13*). As expected from previous results, the majority of OVA-specific OT-1 TCR$^+$ IELs (*14*) bound the TL tetramer (*5*), although this was not the case for the CD8$^+$ splenocytes from the same animals (*11*). Transgenic T cells were cultured with OVA peptide–loaded stimulator cells, or TL-transfected variants (*13*). Similarly to the CD8αα-expressing hybridomas, the presence of TL enhanced antigen-induced IL-2 production by the tetramer-binding IELs (Fig. 3B). Furthermore, increased interferon-γ (IFN-γ) production was observed by intracellular cytokine staining of these antigen-stimulated IELs as well (Fig. 3B), and confirmed by enzyme-linked immunosorbent assay (ELISA) (*11*).

Because TCR transgenic IELs might not be entirely representative of wild-type IELs, we also analyzed polyclonally activated cells from normal mice. Consistent with results seen with the antigen-stimulated TCR transgenic IELs, CD3 stimulated CD8$^+$ normal IELs also showed increased IL-2 and IFN-γ release in the presence of TL (Fig. 3C). It is noteworthy that TL included as tetramers (Fig. 3D) or expressed on bystander cells (*11*)

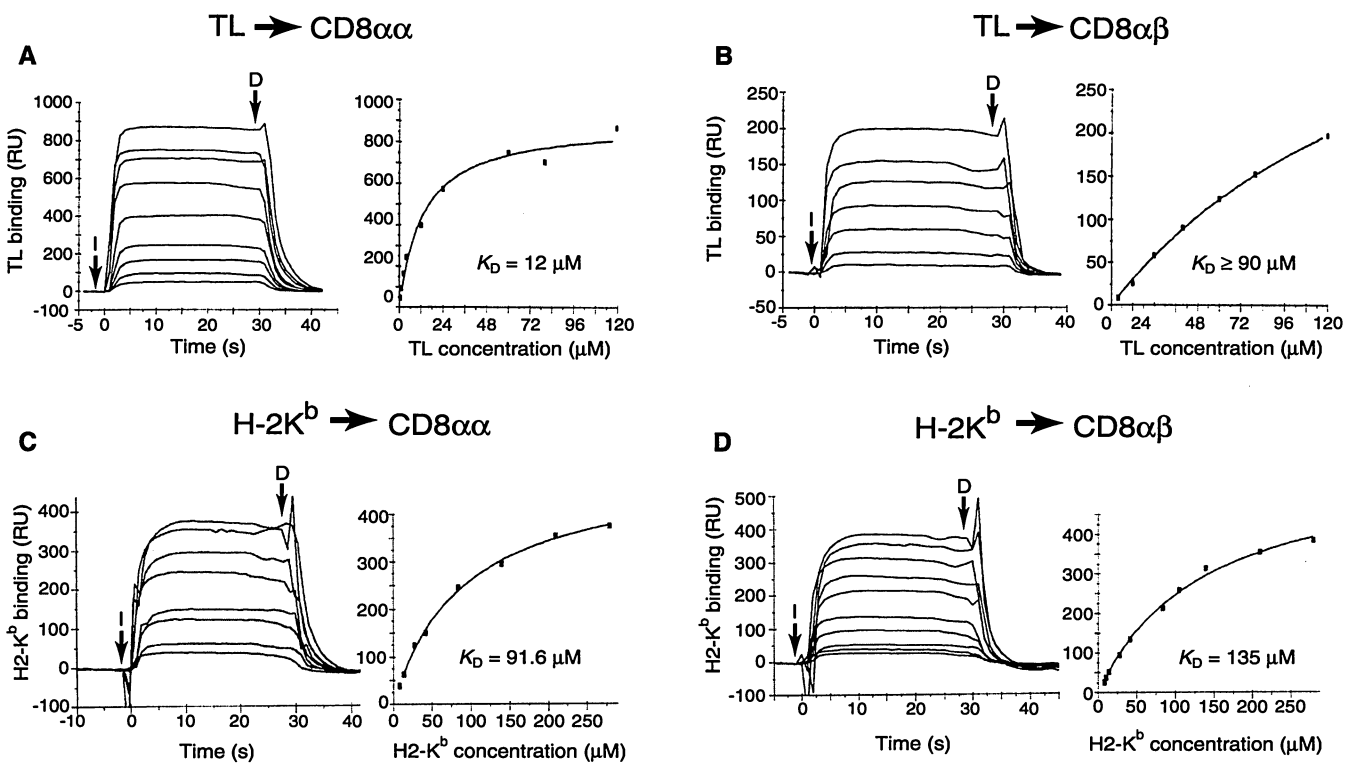

Fig. 2. Surface plasmon resonance measurements of TL and H-2K^b binding to CD8. (**A**) TL binding to immobilized CD8αα (TL→CD8αα). Binding sensograms are on the left and a plot of equilibrium binding as a function of TL concentration is shown on the right. I, injection, D, dissociation phase. Binding of between 0.6 and 120 μM TL to 1500 RU of immobilized CD8αα was analyzed. (**B**) TL binding to immobilized CD8αβ (TL→CD8αβ). Binding of between 4 and 120 μM TL to 1700 RU of immobilized CD8αβ. (**C**) H-2K^b→ CD8αα. H-2K^b heavy chain and β2m were refolded with OVA peptide SIINFEKL by standard methods (*29*). Between 8.4 and 280 μM soluble H-2K^b peptide monomers were passed over a CD8α-coupled chip, and the binding was analyzed as described for TL. (**D**) K^b→ CD8αβ. Binding of soluble H-2K^b to a CD8αβ-coupled chip was as described above. Each measurement is representative data from one of at least two independent experiments.

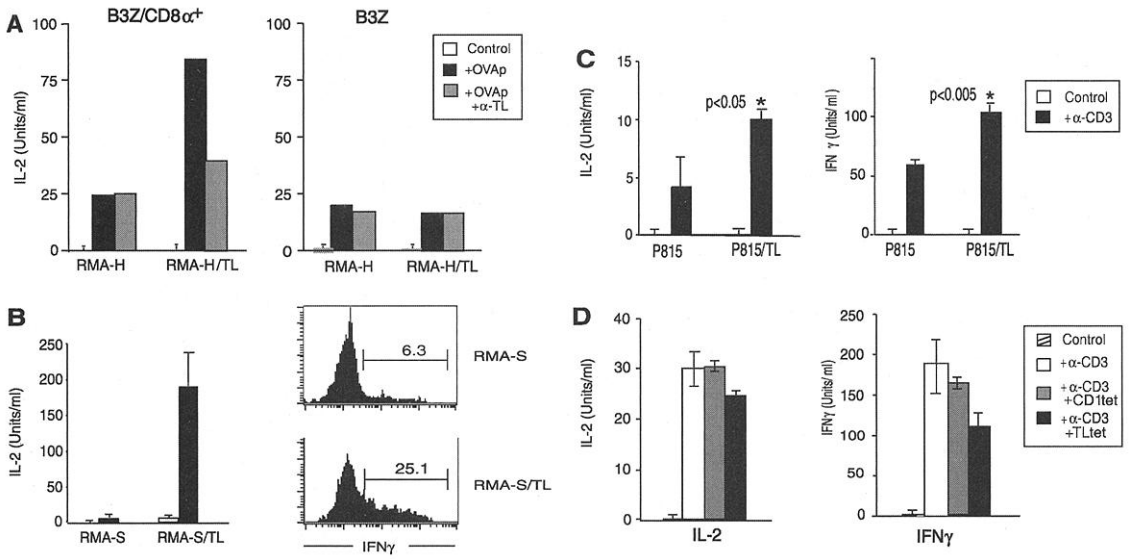

Fig. 3. The TL-CD8αα interaction enhances cytokine production. (**A**) OVA-specific B3Z hybridoma parent cells (*27*), or transfectants expressing CD8α (*27*), were cultured with SIINFEKL peptide loaded (+OVAp) or unloaded (−) RMA-H thymoma cells as stimulators, or TL-transfected RMA-H (RMA-H/TL) cells. For blocking, 10 μg per well of TL-specific antibody was added (+OVAp +α-TL). The amounts of IL-2 were measured by ELISA from the supernatants after 24 hours. The data are representative of one of three independent experiments. (**B**) $CD8^+$ IELs (10^5 per well) purified from OT-1 TCR transgenic mice (*14*) were incubated with 10^5 irradiated and peptide loaded (+OVAp) or unloaded (–) RMA-S or RMA-S/TL stimulator cells. Amounts of IL-2 were measured by ELISA from the supernatants after 72 hours. The bars are the average of two independent experiments. Amounts of intracellular IFN-γ on OT-1 TCR^+ IELs that were purified by magnetic beads with CD8α were measured after 20 hours in vitro stimulation. The data represent one of two independent experiments. (**C**) Purified IELs were incubated with P815 or TL transfectants of these cells (P815/TL) loaded with CD3ε-specific antibody. The amounts of IFN-γ and IL-2 were measured by ELISA after 24 hours in the presence (+α-CD3) or absence (–) of TCR-specific antibody. The bars represent results from one of two independent experiments. (**D**) $CD8\beta^+$ IELs from C57BL/6 mice were purified as described (*14*) and cultured in plates coated with 1 μg/ml purified CD3ε-specific antibody. TL or CD1d tetramers were added at 10 μg per well per day. The amounts of IFN-γ and IL-2 were measured by ELISA after 72 hours culture. The data represent one of two independent experiments.

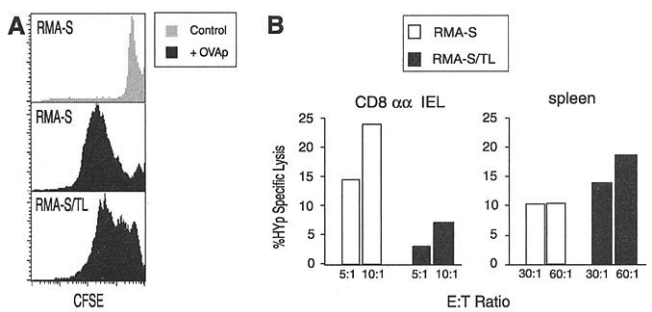

Fig. 4. The TL-CD8αα interaction does not enhance other T cell functions. (**A**) Proliferation. Purified $CD8^+$ IELs (90 to 95% pure) from monoclonal OT-1 TCR transgenic mice were CFSE-labeled and incubated as described (*13*) with either SIINFEKL peptide–loaded stimulator cells (+OVAp), or cells without peptide (–). Proliferation was monitored 3 days later by flow-cytometry analysis, gating on the $CD8\alpha^+$ OT-1^+ ($V\beta5^+$) cells. Data are from one representative experiment of three. (**B**) Cytotoxicity. $CD8\alpha\alpha^+$ IELs of H-Y TCR transgenic RAG $2^{-/-}$ male mice sorted by flow cytometry, and freshly isolated splenocytes were used in a ^{51}Cr-release assay (*13*), with H-Y peptide loaded RMA-S or RMA-S/TL as target cells. Shown is the H-Y antigen–specific lysis only; the data are representative of three experiments.

did not enhance cytokine production, indicating that the CD8αα-mediated modulation of the TCR response occurs only when the stimulator cell expresses both TL and the MHC-peptide complex recognized by that TCR.

After OT-1 IELs and splenocytes were both labeled with 5-carboxy fluorescein diacetate succinimidyl ester (CFSE), they were stimulated in vitro and monitored for their proliferation (*13*). TL expression did not increase the proliferation of OVA-stimulated IELs, and in fact, the proliferation rate was consistently decreased (Fig. 4A). These data indicate that the elevated cytokine production in the presence of TL was not due to increased expansion of the effector population. Next, sorted $CD8\alpha\alpha^+$ IELs from male H-Y TCR transgenic mice (*15*) were used to test the cytotoxic potential of IELs in the presence of TL (*13*). Although surface amounts of D^b were equivalent on peptide-loaded RMA-S/TL or RMA-S cells (*5*), specific killing by the $CD8\alpha\alpha^+$ transgenic IELs of the TL coexpressing target cells was less efficient (Fig. 4B). As expected, unprimed $CD8\alpha\beta^+$ splenocytes showed little cytotoxicity, and this was not affected by TL (Fig. 4B).

CD8αβ acts as a TCR coreceptor by binding simultaneously to the same MHC molecule as the TCR, thereby participating in the TCR complex. By contrast, CD8αα displays poor coreceptor function (*16*). In a number of αβ TCR transgenic systems where the TCR is specific for class I or class II molecules, $CD8\alpha\alpha^+$-expressing T cells are generated, and this has been associated with the presence of agonistic self-peptides (*15*, *17*, *18*). These data suggest that the expression of CD8αα might be correlated with activation, rather than class I specificity of the TCR. Consistent with this, IELs that express superantigen-autoreactive Vβs are present among the $CD8\alpha\alpha^+$ population (*19*). Furthermore, conventional $CD4^+$ T cells acquire CD8αα upon migration to the intestine (*20*), particularly upon chronic activation, as in colitis models (*21*). Collectively, these data suggest that CD8αα expression can be induced or selected for in the intestine regardless of TCR specificity.

These findings allow a reevaluation of hypotheses concerning the role of CD8αα, and they suggest that the effects of CD8αα expression on IELs are due to its interaction with TL. In vivo, the promotion of IELs cytokine release after TCR activation, rather than proliferation or cytotoxicity, may allow adaptation to residence in the single layer of epithelial cells. In this environment, space for clonal expansion is lacking, and excessive tissue destruction by IELs could have detrimental effects on the barrier function of the epithelium. Therefore, we suggest that the interaction of TL with CD8αα on IELs could have important regulatory effects that influence homeostasis, activation, and survival of IELs under the high antigen load of the intestine.

Class I molecules interact with the CD8αβ

coreceptor in conjunction with the TCR. Additionally, by binding to natural killer (NK) receptors, class I molecules can modulate NK activity (*22*, *23*). Here we describe a third pathway by which class I molecules may affect lymphocyte function, by interacting specifically with CD8αα. By binding to TL independently of the TCR MHC specificity, CD8αα acts semiautonomously and not as a TCR coreceptor. This type of interaction may not be exclusive to IELs, as T cells in other tissues also can express CD8αα (*24*, *25*). With the findings presented here, the possibility must now be entertained that CD8αα molecules could have a regulatory function through high-affinity binding to class I molecules.

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12. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
13. *IL-2* release assays were carried out using peptide-pulsed, C57BL/6-derived RMA-H thymoma cells as stimulators, or TL transfectants of these cells and OVA peptide-specific B3Z hybridoma cells and CD8α$^+$-transfected variants (*27*) as the effector cells. CD8$^+$ IELs from OT-1 TCR transgenic mice (*14*) were purified (>90%) with magnetic beads (MACS) coated with CD8α mAb (MiltenyiBiotech, GmbH, Germany) according to the manufacturer's guidelines. Cytokine release by IELs was measured in cultures using OVA peptide–pulsed RMA-S thymoma cells deficient in transporter associated with antigen processing (TAP), or TL transfectants of these cells (*6*). Purified OT-1 IELs were labeled with CFSE and 7.5 to 10 × 10^4 per well were incubated with 10^5 RMA-S cells, RMA-S TL transfectants, or the same cells loaded with OVA peptide. Proliferation was monitored 3 days later by flow cytometry. Sorted CD8αα$^+$ IELs and CD8$^+$ splenocytes of H-Y TCR transgenic RAG $2^{-/-}$ male mice (*15*) were used in a 4-hour ^{51}Cr-release assay, with 5 × 10^3 KCSRNRQYL (H-Y) peptide–loaded RMA-S or RMA-S/TL target cells (*12*). CD8$^+$ normal IELs were sorted using the CD8β antibody, to prevent triggering of CD8αα. Effector cells (10^5 per well) were incubated with 10^5 of CD3ε-specific, antibody–loaded, Fc receptor–positive P815 mastocytoma cells (ATCC), or TL transfectants variants. Alternatively, IELs were cultured in wells coated with 1 μg/ml anti-CD3ε-specific antibody, TL, or CD1d tetramers were added at 10 μg per well per day, and cytokines were measured by ELISA after 72 hours.
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RGS-PX1, a GAP for Gα$_s$ and Sorting Nexin in Vesicular Trafficking

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Heterotrimeric GTP–binding proteins (G proteins) control cellular functions by transducing signals from the outside to the inside of cells. Regulator of G protein signaling (RGS) proteins are key modulators of the amplitude and duration of G protein–mediated signaling through their ability to serve as guanosine triphosphatase–activating proteins (GAPs). We have identified RGS-PX1, a Gα$_s$-specific GAP. The RGS domain of RGS-PX1 specifically interacted with Gα$_s$, accelerated its GTP hydrolysis, and attenuated Gα$_s$-mediated signaling. RGS-PX1 also contains a Phox (PX) domain that resembles those in sorting nexin (SNX) proteins. Expression of RGS-PX1 delayed lysosomal degradation of the EGF receptor. Because of its bifunctional role as both a GAP and a SNX, RGS-PX1 may link heterotrimeric G protein signaling and vesicular trafficking.

Heterotrimeric G proteins relay extracellular signals initiated by hormones, neurotransmitters, chemokines, and sensory stimuli through G protein–coupled receptors to intracellular effectors and trigger a variety of physiological responses (*1*, *2*). Receptor activation causes dissociation of Gα subunits from Gβγ dimers and subsequent regulation of downstream effectors. Members of the RGS protein family serve as GAPs that attenuate G protein–mediated signal transduction by binding to Gα subunits through a conserved RGS domain and accelerating GTP hydrolysis of Gα subunits (*3*).

The RGS proteins characterized to date are GAPs for G_i, G_q, or $G_{12/13}$ classes of G proteins, but no RGS GAP for Gα$_s$ has been found. To identify RGS proteins that might serve as GAPs

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