Toso, a Functional IgM Receptor, Is Regulated by IL-2 in T and NK Cells

Yousuke Murakami, Sriram Narayanan, Su Su, Richard Childs, Konrad Krzewski, Francisco Borrego, Jennifer Weck and John E. Coligan

*J Immunol* published online 6 June 2012
http://www.jimmunol.org/content/early/2012/06/06/jimmunol.1200840
Toso, a Functional IgM Receptor, Is Regulated by IL-2 in T and NK Cells

Yousuke Murakami,* Sri Ram Narayanan,* Su Su,† Richard Childs,† Konrad Krzewski,* Francisco Borrego,‡ Jennifer Weck,* and John E. Coligan*

We find that the cell surface receptor Toso is dramatically downregulated by IL-2. Toso expression is induced in transfected cells, hence more properly designated FcRm. Despite these reports, the function of Toso/FcRm is not inherently an antiapoptotic molecule. The antiapoptotic effects of Toso were not observed when either Fasl or an IgG mAb to Fas were used to induce apoptosis. The evidence indicating that Toso is an IgM receptor, and hence more properly designated FcRm, is supported by its genetic location next to the genes for the polymeric Ig receptor and the FcR/μR (14). Despite these reports, the function of Toso/FcRm remains a subject of active debate. In this regard, a recent publication (16) showed that Toso is an antiapoptotic molecule that does not bind IgM and functions by recruiting the death adapter FADD to a Toso–RIP1 protein complex. Our interest in Toso/FcRm began when we identified it as a gene product that is dramatically downregulated in NK cells treated with IL-2, which led us to postulate that as an antiapoptotic molecule, its downregulation might facilitate AICD. We have followed with interest the controversy regarding Toso/FcRm function and in this study present data that indicate Toso/FcRm binds IgM and delivers an activating signal to NK cells. Moreover, we demonstrate that IL-2 downregulates Toso/FcRm expression by both NK and T cells and that this suppression is a dynamic and reversible process. We further show that TCR activation of CD4 T cells results in downregulation of Toso/FcRm. In accord, we found that, relative to naive T cells, Toso/FcRm levels are low on effector and central memory T cells, which correlates with their activation status. However, in contrast to earlier studies showing that Toso/FcRm is an antiapoptotic molecule (12, 13, 16), upon overexpression of Toso/FcRm in Jurkat T and peripheral blood NK cells, we were not able to inhibit Fas-mediated apoptosis induced by CD16.

*Receptor Cell Biology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852; †Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; and ‡Laboratory of Molecular and Developmental Immunology, Division of Monoclonal Antibodies, Center for Drug Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892

Received for publication March 15, 2012. Accepted for publication May 8, 2012.

This work was supported by the intramural program of the National Institute of Allergy and Infectious Diseases and the intramural program of the National Heart, Lung, and Blood Institute.


Address correspondence and reprint requests to Dr. John E. Coligan, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Tabinbrook IL, Room 205, 12441 Parklawn Drive, Rockville, MD 20852. E-mail address: jcoligan@niddk.nih.gov

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; ADCC, antibody-dependent cell-mediated cytotoxicity; AICD, activation-induced cell death; FADD, Fas-associated death domain protein; Fasl, Fas ligand; FcαR, human pentameric FcαR fragment; IRES, internal ribosome entry site; mIgM, mouse IgM; TCM, central memory; TEM, effector memory.
by FasL. We anticipate our work will extend the recognition of Toso/FcγR as an IgM receptor capable of activating signaling molecules and whose expression alone is not inherently antiapoptotic.

Materials and Methods

Cell culture

NK, CD4, and CD8 T cells were isolated from human peripheral blood using stem cell isolation kits (NK cells) or Miltenyi Biotec MACS columns (T cells) and their purity (≥95%) determined by staining with anti-CD3 mAb (eBioscience) and anti-CD56 mAb (BD Biosciences) for NK cells and anti-CD3 mAb and anti-CD4 mAb (eBioscience) or anti-CD8 mAb (eBioscience) for CD4 and CD8 T cells, respectively. Primary T cells were cultured in IMDM containing 10% human AB serum (Valley Biomedical) and 100 U/ml IL-2. NK cells were cultured as described for each assay.

Lentiviral vector construction

Lentiviral vectors expressing human Toso were constructed from the parent vector (pCL20c MSCV GFP), kindly provided by Dr. Arthur Nienhuis (St. Jude Children’s Research Hospital, Memphis, TN). First, an internal ribosome entry site (IRES) was inserted using Bst1107I and SacII sites. A Toso cDNA fragment flanked with ClaI and AgeI sites was PCR amplified, Clal digested, and inserted into pCL20c MSCV IRES-GFP, thus generating pCL20c MSCV Toso-IRES-GFP. A pCL20c MSCV Toso-T2A-GFP vector was generated in the same way. Human Toso cDNA was also cloned into the lentiviral vector, pcDH, which contains a puromycin resistance gene, allowing selection of positive clones. All vector constructs were confirmed by DNA sequence analysis.

Vector production and transduction

293T cells were cotransfected with viral packaging encoding constructs and either of the Toso lentiviral expression vectors described earlier. Vector particles were harvested after 24 h, cleared by centrifugation, filtered, snap frozen in aliquots, and stored at −80°C. For lentivirus (LV) transduction into NK cells, freshly isolated primary NK cells were cultured with irradiated (100 Gy) EBV-transformed B cells as feeder cells in IMDM containing 10% human AB serum and 500 U/ml of IL-2 as previously described (17). Jurkat T or NK cells were seeded with retronectin (Takara Bio) and transduced (95% transduction efficiency as measured by GFP) with pCL20c MSCV Toso, NK cells, freshly isolated primary NK cells were cultured with retronectin (Takara Bio) and transduced (100 Gy) EBV-transformed B cells as feeder cells in IMDM containing 10% human AB serum and 10% human AB serum (Valley Biomedical) and anti-CD3 mAb and anti-CD4 mAb, conjugated to FITC, PE, and allophycocyanin, respectively (all from eBioscience). The cells were incubated on ice for 30 min after which they were washed extensively with PBS containing 1% human serum and sorted using a FACSAria sorter (Beckton Dickinson). Stimulation of CD4 T cells was done using plate-bound anti-human CD3 mAb (1 μg/ml; BD Biosciences) for the indicated times.

Real-time PCR analysis

NK cells were isolated from PBMCs and cultured in IMDM with 10% human AB serum with or without IL-2 as indicated. All samples for RNA isolation were stored at −80°C in RNALater (Ambion). RNA isolation was carried out using the RNeasy miniPCR kit (Ambion) and cDNA produced using the Qu-script kit (Quanta Biosciences). Real-time PCR was done on a Roche LC480 cycler using Lightcycler 480 SYBR green I master mix (Roche Diagnostics). Primers for real-time PCR measurement of human Toso, actin, and 18S rRNA were purchased from Qiagen. All reactions were done in triplicate, and averages were used to calculate the relative levels of each mRNA. Relative quantification of the target genes was determined using the second derivative maximum using the Roche Lightcycler software and calculating the fold changes over the 18S rRNA or actin transcript levels. Melting curve analysis was performed to ensure that only one product was amplified.

STAT5 and PI3K inhibitor studies

Freshly isolated human peripheral blood NK cells were preincubated for 30 min in IMDM plus 10% human serum with FcγRII (IgG2a) and FcγRIII (IgG3) mAbs (Sigma-Aldrich) at various concentrations as indicated. IL-2 (500 U/ml) was added, and cells were cultured for 24 h before harvesting, washing, and storing in RNAlater. RNA was isolated, cDNA was synthesized, and the relative levels of Toso mRNA were quantified by normalizing to the level of 18S rRNA transcripts using real-time PCR as described earlier.

Measurement of cytotoxicity

NK cells were isolated and maintained in X-vivo medium with 10% Ig-depleted serum and 500 U/ml IL-2 for 1 wk prior to culturing in X-vivo medium plus 10% Ig-depleted medium without IL-2 overnight. P815, K562, or SK-OV3 target cells were labeled with DELFIA BATDA (TDA) cytotoxicity reagents (PerkinElmer). Cells were washed three times in PBS and resuspended in X-vivo medium with 10% Ig-depleted serum. P815 cells were incubated for 30 min at room temperature with anti-human CD6 mAb (100 ng/ml; negative control) or anti-human Toso mAb. CD8 T cells were stained with anti-human IgM mAb (BD Biosciences), anti-CD8 mAb (eBioscience), anti-human Toso mAb, or control IgG (100 ng/ml; BD Biosciences). SK-OV3 cells were treated with anti-HER2 (ErbB2) mAb trastuzumab (50 ng/ml) then spun down, resuspended in medium, and plated at 104 cells/well in triplicate in 96-well round-bottom plates. NK cells were incubated with mlgM (10 μg/ml), mlgM plus anti-HER2 mAb or the IgM triantibody Lewis Ab (10 μg/ml; Abcam) at 37°C for 30 min, then added to wells at the indicated E:T ratios. After 2-h incubation, the supernatant was collected, and release of TDA was measured according to the manufacturer’s instructions using a Wallac Victor plate reader (PerkinElmer).

Western blots

For immunoprecipitations, YTS cells overexpressing Toso were lysed in ice-cold lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 2 mM EDTA, 5% glycerol, X-100 including protease inhibitor mixture; Sigma-Aldrich) at the indicated concentrations for 10 h. Annexin V staining was performed using the BD Biosciences kit according to the manufacturer’s protocol. Apoptosis of the transduced NK cells, sorted into GFP+ or GFP− populations, was induced using anti-human Fas mAb clone 2R2 or FasL and cross-linking enhancer (Alexis Biochemicals) at the concentrations indicated in the figure legends for 12 h. For analysis of L- transduced NK cells, annexin V–allophycocyanin was used according to the manufacturer’s protocol.

T cell sorting

For sorting CD4 T cells into naive and memory cells, isolated CD4 T cells were washed with PBS containing 1% human serum and stained with anti-human CD4 mAb, CD45RO mAb, and CD62L mAb, conjugated to PE, and allophycocyanin, respectively (all from eBioscience). The cells were incubated on ice for 30 min after which they were washed extensively with PBS containing 1% human serum and sorted using a FACSAria sorter (Beckton Dickinson). Stimulation of CD4 T cells was done using plate-bound anti-human CD3 mAb (1 μg/ml; BD Biosciences) for the indicated times.

For sorting CD8 T cells into naive and memory cells, isolated CD8 T cells were washed with PBS containing 1% human serum and stained with anti-human CD8 mAb, conjugated to FITC, PE, and allophycocyanin, respectively (all from eBioscience). The cells were incubated on ice for 30 min after which they were washed extensively with PBS containing 1% human serum and sorted using a FACSAria sorter (Beckton Dickinson). Stimulation of CD8 T cells was done using plate-bound anti-human CD3 mAb (1 μg/ml; BD Biosciences) for the indicated times.

Measurement of cytotoxicity

NK cells were isolated and maintained in X-vivo medium with 10% Ig-depleted serum and 500 U/ml IL-2 for 1 wk prior to culturing in X-vivo medium plus 10% Ig-depleted medium without IL-2 overnight. P815, K562, or SK-OV3 target cells were labeled with DELFIA BATDA (TDA) cytotoxicity reagents (PerkinElmer). Cells were washed a total of 500 U/ml was added, and cells were cultured for 24 h before harvesting, washing, and storing in RNAlater. RNA was isolated, cDNA was synthesized, and the relative levels of Toso mRNA were quantified by normalizing to the level of 18S rRNA transcripts using real-time PCR as described earlier.

STAT5 and PI3K inhibitor studies

Freshly isolated human peripheral blood NK cells were preincubated for 30 min in IMDM plus 10% human serum with N-(4-oxo-4H-chromen-3-yl) methylene) nitrosohydrazide (STATA5 inhibitor; EMD Biosciences) or wortmannin (PI3K inhibitor; Sigma-Aldrich) at various concentrations as indicated. IL-2 (500 U/ml) was added, and cells were cultured for 24 h before harvesting, washing, and storing in RNAlater. RNA was isolated, cDNA was synthesized, and the relative levels of Toso mRNA were quantified by normalizing to the level of 18S rRNA transcripts using real-time PCR as described earlier.
with protein G Dynabeads (Invitrogen) at 4°C. The beads were washed four times with lysis buffer and the proteins eluted with NuPAGE LDS sample buffer (Invitrogen). Precipitates were separated by 4–12% gradient Bis-Tris gels (Invitrogen) and transferred to polyvinylidene fluoride. Blots were incubated overnight at 4°C with anti-human Toso mAb, anti-PLCγ2 polyclonal Ab (Cell Signaling), or anti-p–Thr Tyr mAb (clone 4G10; Millipore), followed by incubation with HRP-conjugated secondary Ab (Santa Cruz Biotechnology). To detect signaling molecules, 1 × 10^5 to 2 × 10^5 YTS cells were cultured in cRPMI without serum overnight, then collected and resuspended in 25 μl serum-free cRPMI prior to treatment. Cultured NK cells were isolated from PBMCs and cultured for 1 wk in X-vivo medium with 10% Ig-depleted serum and 500 IU/ml IL-2 before culturing in serum-free X-vivo medium without IL-2 overnight. Fresh NK cells were isolated from PBMCs and cultured in X-vivo medium with neither serum nor IL-2 for 14–16 h. In either case, 5 × 10^6 NK cells were collected and resuspended in 15 μl X-vivo medium prior to treatment. YTS cells and NK cells were treated with PBS (control), 5 μg/ml mIgM, 5 μg/ml anti-mIgM Ab, or both for 30 min at 37°C. Ice-cold lysis buffer (100 mM Tris pH 7.4, 1% Nonidet P-40, 200 mM NaCl, 2mM EDTA) was added and cells lysed for 30 min prior to centrifugation to remove cellular debris. Supernatants were denatured with 4× NuPage LDS buffer with DTT, run on gels, transferred to nitrocellulose, and probed with a rabbit anti–pERK1/2 Ab (Cell Signaling), then reprobed with a polyclonal Ab to total ERK1/2 (Cell Signaling). The blots were developed using ECL plus (Amersham Biotech) or Pierce Pico Western substrate. The integrated density of each band was measured using the gel analysis function of ImageJ (version 1.44a), normalized to the loading control, and compared with the untreated sample (value = 1).

**Statistical analysis**

For all experiments, statistical analysis and graphing were done using the GraphPad Prism 5 software. Two-tailed, unpaired Student t test with 95% confidence interval was used to compare the statistical significance of the differences observed between treatments. In some cases, the paired t test was used for analysis as indicated in the figure legends. Significance is indicated as follows: *p < 0.05, **p < 0.01, and ***p < 0.005.

**Results**

**Toso links IgM**

We analyzed the ability of Toso to bind IgM using Jurkat T and YTS NK cell lines overexpressing Toso, as well as NK cells isolated from PBMCs. In our experiments, we used mIgM because mIgM binds human Toso better than human IgM does (15). Empty LV-transduced cells did not express Toso on the cell surface, as measured by flow cytometry, whereas the cells transduced with LV-Toso expressed the receptor (Fig. 1A). mIgM binding to these cells was detected by staining with mIgM plus anti-mIgM Ab PE or by treating cells directly with allophycocyanin-labeled mIgM (Fig. 1A). [Unlike human sera, we found that any IgM present in FCS did not block the mIgM binding to cells (data not shown)]. As circulating NK cells (both CD56bright and CD56dim) express Toso (Fig. 1B, top) and are constantly exposed to serum IgM, we tested if NK cells in PBMCs have IgM bound to them. PBMCs were collected and gated on NK-specific markers (CD3- and CD56+). Staining with an anti-human IgM Ab indicates IgM is bound to circulating NK cells (Fig. 1B, bottom). To verify the specificity of the IgM binding, NK cells were isolated from PBMCs, cultured in X-vivo medium with Ig-depleted serum plus IL-2 for 7 d, then in the same medium without IL-2 overnight. These NK cells bound mIgM–allophycocyanin Ab and expressed Toso on the cell surface (Fig. 1C). NK cells exhibited essentially no binding to IgM when pretreated with FcγR, indicating that the binding is specific (Fig. 1C). Toso expression on these same NK cells is also shown (Fig. 1C). We have found that mIgM binding to NK cells correlates with Toso expression by these cells (see Fig. 3B). These results demonstrate that Toso is expressed on NK cells and is likely the receptor responsible for IgM binding. Thus, throughout the remainder of this article, we refer to the receptor as Toso/FcγR.

**Toso/FcγR expression does not inhibit apoptosis in primary human NK or Jurkat T cells**

As mentioned, recent publications differ in their findings as to whether Toso/FcγR expression is inherently apoptotic (16) or not (15). To investigate the antiapoptotic activity of Toso/FcγR, we treated control Jurkat cells or Jurkat overexpressing Toso/FcγR (see Fig. 1A) with various concentrations of FasL or anti-Fas Ab. Both control Jurkat and Toso/FcγR-overexpressing cells were cultured in cRPMI with Ig-depleted serum for 1 wk to remove any IgM. Under no circumstances did Toso/FcγR affect FasL-induced apoptosis, as evidenced by staining with 7-aminoactinomycin D (7-AAD) and annexin V, nor did it affect IgG anti-Fas Ab-mediated apoptosis (Fig. 2A, 2B), even in the presence of mIgM. Only IgM anti-Fas Ab-treated cells exhibited reduced apoptosis, and this occurred in the presence or absence of excess mIgM (Fig. 2A, 2B). The effect of the IgM anti-Fas Ab is not due to the IgM isotype as mIgM (10 μg/ml) does not interfere with the antiapoptotic effect of FasL or IgG anti-Fas Ab (Fig. 2B). This suggests that the antiapoptotic effect of IgM anti-Fas Ab is due to the cross-linking of Toso/FcγR and Fas (see Discussion). To verify further that Toso/FcγR expression is not inherently antiapoptotic, we transduced primary NK cells, cultured and expanded in IMDM with human serum and IL-2, with a Toso/FcγR-expressing LV that also encodes for GFP. Cells transduced with an LV expressing GFP alone were used as control. Transduction with the Toso/FcγR-GFP LV clearly produced a population of cells enriched for the surface expression of Toso/FcγR compared with NK cells transduced with the GFP LV control (Fig. 2C, left). After stimulation of Fas-mediated apoptosis using IgG anti-Fas mAb or FasL, we observed that the level of apoptosis, as measured by annexin V staining, was similar in cells transduced with GFP LV or Toso/FcγR-GFP LV (Fig. 2C, right). Thus, Toso/FcγR overexpression cannot rescue human NK cells expanded in vitro with IL-2 from Fas-mediated cell death, further indicating that under these conditions, Toso/FcγR is not an antiapoptotic molecule.

**Toso/FcγR expression by NK cells is regulated by IL-2 through a STAT5-dependent pathway**

We found that NK cells isolated from human PBMCs (cultured in IMDM plus human serum) with IL-2 for 48 h had a dramatic (80–90%) downregulation of Toso/FcγR transcript levels compared with cells cultured without IL-2 (Fig. 3A, left). This is accompanied by marked (~50%) decrease in cell surface staining using an anti-Toso mAb (Fig. 3A, right). Thus, IL-2 downregulates the expression of Toso/FcγR mRNA and cell surface expression by NK cells. We found that mIgM does not bind to NK cells cultured in X-vivo medium with Ig-depleted serum when IL-2 is present, yet when NK cells are cultured in medium without IL-2 overnight, mIgM binds (Fig. 3B). Thus, mIgM binding correlates with Toso/FcγR expression by NK cells. When culturing NK cells over a long period of time in IMDM with human serum, we observed that Toso/FcγR expression was maintained at low levels in medium containing IL-2. When NK cells cultured in this manner are washed and placed in fresh medium supplemented with 10% human serum, but lacking IL-2 for 16 h, Toso/FcγR expression increased (~5-fold in mRNA and 2-fold in cell surface expression) (Fig. 3C). Upon binding of IL-2, signaling through the hetero- trimeric IL-2Rα/β/γ activates multiple signaling pathways, including that of PI3K and JAK3–STAT5 (18, 19). To determine if PI3K or JAK3–STAT5 is involved in the IL-2–mediated downregulation of Toso/FcγR, we used specific inhibitors for PI3K or JAK3–STAT5 to block IL-2–dependent signaling from these pathways.
FIGURE 1. Toso/FcµR binds IgM. (A) Jurkat T or YTS cells transduced with control LV or Toso-encoding LV (LV-Toso) were stained with anti-Toso mAb plus anti-IgG Ab PE or allophycocyanin-conjugated mIgM (mIgM-APC) or mIgM plus anti-IgM Ab PE and analyzed by flow cytometry. (B) Top panel, Freshly isolated PBMCs were stained with anti-Toso, anti-CD3, and anti-CD56 mAbs. Subsets of NK cells were recognized by CD56 bright or CD56 dim populations and analyzed for Toso expression. Right panel, Average from four donors; error bars indicate SEM. Statistical analysis was done by two-tailed unpaired Student t test. Bottom panel, Freshly isolated PBMCs were stained with anti-human IgM µ-chain mAb (anti-IgM) or anti-Toso mAb and gated on NK cells. These histograms are representative of three experiments using cells from different donors. (C) NK cells that had been cultured in X-vivo medium with Ig-depleted serum were preincubated with or without FcµR then stained with allophycocyanin-conjugated mIgM (mIgM-APC) and analyzed by flow cytometry. Expression of Toso/FcµR on these NK cells is also shown. This is one representative of three experiments. **p < 0.01.
FIGURE 2. TosoFcμR is not antiapoptotic. (A) Jurkat T cells transduced with control LV or LV-TosoFcμR were stimulated with IgM anti-Fas mAb or FasL, at indicated concentrations for 10 h and analyzed for apoptosis by annexin V and 7-AAD staining. One representative dot plot and bar graphs from three independent experiments are shown. Error bars indicate SEM. Statistics were calculated using unpaired, two-tailed Student t test. (B) Jurkat T cells transduced with control LV or LV-TosoFcμR were untreated or stimulated with IgM anti-Fas mAb (10 ng/ml), FasL (100 ng/ml), or IgG3 anti-Fas mAb (50 ng/ml) in the absence or presence of mIgM (10 μg/ml) and the percentage of annexin V+ stained cells was determined by flow cytometry. Bar graphs indicate data from at least four experiments. (C) Left panel, NK cells transduced with GFP-LV or Toso/FcμR–GFP-LV were stained with anti-Toso mAb. Toso/FcμR expression was analyzed by flow cytometry and shown from a representative donor. Right panel, Toso/FcμR-transduced NK cells were sorted into GFP+ and GFP− cells and analyzed for Fas-mediated apoptosis after culturing with IgG3 anti-Fas mAb (50 ng/ml) or recombinant FasL (100 ng/ml) and cross-linking Ab (1 μg/ml) for 12 h. Apoptotic cells were determined by staining with annexin V–allophycocyanin using flow cytometry. Data shown are the average from three different donors, and error bars indicate SEM. *p < 0.05.
pathways. Freshly collected NK cells were preincubated with wortmannin (PI3K inhibitor) or a STAT5 inhibitor, then cultured in medium with or without IL-2 for 24 h prior to mRNA isolation. STAT5 inhibition completely blocked Toso/FcμR downregulation in a dose-dependent manner, whereas wortmannin could not rescue the expression of Toso/FcμR mRNA levels (Fig. 3D). Thus, the JAK–STAT pathway but not the PI3K pathway is involved in IL-2–mediated downregulation of Toso/FcμR.

IL-2 also regulates Toso/FcμR expression in human CD4 and CD8 T cells

Because Toso/FcμR was originally discovered in T cells (12), and because AICD is a pathway that has been shown to be important in the homeostasis of peripheral T cells (20), we cultured CD4 T cells in wells coated with plate-bound anti-CD3 mAb. Upon activation with anti-CD3 mAb, Toso/FcμR mRNA levels were reduced to levels similar to that regulated by IL-2 (Fig. 4B).

We hypothesized that naïve T cells, which are not activated, should have a higher level of Toso/FcμR expression than that of effector T cells. To test this hypothesis, we examined Toso/FcμR expression on central memory (T CM) and effector memory (T EM) CD4 T cells that were isolated from PBMCs. Central and effector memory populations have been defined by the expression of CD45RO and CD62L markers (21), as well as CCR7 in combination with CD45RA (22). Regardless of the markers used to identify T cell subsets, we observed that the T EM subset had significantly lower levels of Toso/FcμR expression compared with those of naive or T CM cells (Fig. 4C). These results support the hypothesis that activation and/or signaling leading to the differentiation of T cell subsets in vivo is accompanied by the down-regulation of Toso/FcμR.

We next examined if in vitro treatment of the various CD4 T cell subsets with activating stimuli, IL-2 or anti-CD3 mAb, would
FIGURE 4. Regulation of Toso/FcγR expression on human CD4 and CD8 T cells. (A) Human peripheral blood CD4 and CD8 T cells were isolated and cultured in the presence or absence of IL-2 (100 IU/ml) in complete IMDM overnight, and Toso/FcγR mRNA expression was quantified by real-time PCR. The results shown are from four donors, and the data are normalized to 18S rRNA expression. (B) Human CD4 T cells from PBMCs were stained for CD45RO and CCR7 and CD45RA (bottom), and the cell surface expression of Toso/FcγR in the naive and memory subsets defined by these markers was determined by staining with anti-FcγR mAb followed by PE-conjugated anti-mouse secondary Ab. Data shown are geometric mean fluorescence intensity of anti-Toso/FcγR staining for six individual donors, each represented by a symbol on the scatterplot. Statistical analysis was done by two-tailed unpaired Student t test. **p < 0.01. (C) Human CD4 T cells isolated from peripheral blood were stained for CD45RO and CCR7 and CD45RA (top) or CCR7 and CD45RA (bottom), and the naive and memory subsets were sorted by FACS. The expression of Toso/FcγR mRNA levels were higher in freshly isolated naive cells compared with those of the T EM cells (Fig. 4D, bottom). At 24 h, we again analyzed Toso/FcγR expression by real-time PCR, and although T EM cells exhibited the greatest level of downregulation (∼65%), T CM cells also showed a marked reduction (∼30%) after IL-2 treatment or anti-CD3 mAb stimulation (Fig. 4D, bottom). In the case of naive T cells, although a reduction in Toso/FcγR mRNA levels was observed, particularly in anti-CD3-treated cells compared with the untreated cells, the difference was not found to be significant (Fig. 4D, bottom).

Toso/FcγR does not mediate NK cell cytotoxicity

As we have observed that Toso/FcγR is not an antiapoptotic molecule and that it specifically binds IgM, we sought to ascribe a function to this receptor in NK cells. Our first course of action was to determine if Toso/FcγR could mediate cytotoxic activity. All NK cells used in these cytotoxic assays were cultured in X-vivo medium with Ig-depleted serum and IL-2 for 1 wk, then washed and cultured in media containing Ig-depleted serum without IL-2 overnight prior to incubation with target cells. NK cells exhibit Ab-dependent cell-mediated cytotoxicity (ADCC), which is mediated by the IgG receptor CD16. We tested if Toso/FcγR could mediate a similar response; that is, does ligation of this IgM receptor by target cell ligands trigger NK cell killing of target cells? NK cells were incubated with IgM anti-Lewis Ag Ab then added to wells containing SK-OV3 cells that express the Lewis Ag. NK cells were unable to kill targets (Fig. 5A, top left). Flow cytometry verified the NK cells-expressed Toso/FcγR and that SK-OV3 target cells could bind the IgM anti-Lewis Ab (Fig. 5A, bottom). In contrast, ADCC utilizing CD16 recognition of IgG anti-HER2 Ab-coated SK-OV3 target cells was successful (Fig. 5A, top right). Redirected lysis of P815 cells through their expression of Fc receptors is a common assay for determining if a given receptor expressed by NK cells can mediate cytotoxic function. To examine if Toso/FcγR could mediate redirected lysis, NK cells were incubated with P815 target cells in the presence of time PCR assay. Data shown are from five independent experiments, and the error bars indicate SEM. Statistical analysis was done by two-tailed unpaired Student t test. *p < 0.05. (D) Human CD4 T cells from PBMCs were stained for CD45RO and CD62L (top) or CCR7 and CD45RA (bottom), and the cell surface expression of Toso/FcγR in the naive and memory subsets defined by these markers was determined by staining with anti-Toso/FcγR mAb followed by PE-conjugated anti-mouse secondary Ab. Data shown are geometric mean fluorescence intensity of anti-Toso/FcγR staining for six individual donors, each represented by a symbol on the scatterplot. **p < 0.01, ***p < 0.001. (D) Top panel, Human CD4 T cells isolated from peripheral blood were stained for CD45RO and CD62L, and the naive and memory subsets were sorted by FACS. The expression of Toso/FcγR mRNA levels was analyzed by real-time PCR, and the data are normalized to 18S rRNA expression. Data shown are mean values of triplicates from six individuals each represented by a symbol on the scatterplot. **p < 0.01, ***p < 0.001. (D) Bottom panel, Sorted cells were stimulated with plate-bound anti-CD3 mAb (1 μg/ml) or IL-2 (100 IU/ml) and analyzed for Toso/FcγR expression by real-time PCR (after 24 h). Data shown are from seven independent experiments, and the error bars indicate SEM. Statistical analysis was done using two-tailed unpaired Student t test comparing each treatment to untreated samples (− IL-2). *p < 0.05.
FIGURE 5. Toso/FcR does not mediate NK cytotoxicity. (A) Top panel, NK cells were incubated with IgM anti-Lewis Ag Ab (10 μg/ml), then mixed with SK-OV3 target cells (left). SK-OV3 target cells were incubated with an IgG anti-HER2 mAb (50 ng/ml), then incubated with anti-IgM mAb (1 μg/ml). Bottom panel, Flow cytometry illustrates the expression level of Toso/FcR on NK cells (left) and binding of the IgM anti-Lewis Ab to SK-OV3 cells (right). (B) Top panel, P815 cells were pretreated with anti-Toso mAb (1 μg/ml; left) or anti-CD16 mAb (100 ng/ml; right) then incubated with NK cells. Bottom panel, Flow cytometry shows expression of Toso/FcR by NK cells (left) and binding of anti-Toso mAb to P815 cells (right). (C) Top panel, Anti-IgM mAb (1 μg/ml)-coated P815 cells were incubated with NK cells in the presence or absence of mlgM (10 μg/ml). Bottom panel, Flow cytometry demonstrates P815 cells are able to bind allophycocyanin-conjugated mlgM (IgM-APC) via anti-IgM mAb. The graphs display representative results and are consistent with data from at least three donors.

Binding of IgM to Toso/FcR on NK cells initiates intracellular signaling

We then sought to determine if Toso/FcR could signal at all upon ligation. YTS cells overexpressing Toso/FcR were cultured in cRPMI with Ig-depleted serum, treated with mlgM, and lysates were immunoprecipitated using an anti-Toso mAb. Immunoprecipitates were separated on a gel and transferred to a membrane that was probed with an anti-phosphotyrosine mAb. A specific phospho-protein band of ~150 kDa was noted and confirmed to be PLCγ2 by using a specific Ab to PLCγ2 (Fig. 7A). Moreover, the cytoplasmic tail of Toso/FcR contains at least three consensus sites specific for binding to molecules that modulate the ERK signaling pathway as determined by Prosite scanning (Eukaryotic Linear Motif resource); therefore, we tested the ability of Toso/FcR to augment activation of NK cells that was mediated by other NK cell receptors. We thus tested the ability of Toso/FcR to augment CD16-mediated ADCC. The incubation of NK cells with SK-OV3 target cells coated with anti-HER2 mAb, which binds and activates CD16, resulted in target cell killing. Neither inclusion of mlgM (to ligate Toso/FcR) nor the IgM anti-Lewis mAb resulted in augmentation of this killing (Fig. 6A). To test if Toso/FcR modifies NK cell cytotoxicity in a redirected lysis assay, anti-CD16 mAb-coated P815 target cells were incubated with NK cells. Whereas NK cells were able to kill these target cells, inclusion of mlgM to ligate Toso/FcR did not alter this killing (Fig. 6B). We also tested if Toso/FcR could alter NK cytotoxicity mediated by the activating receptor NKG2D. P815 cells that had been coated with anti-NKG2D mAb were incubated with NK cells. Although the NK cells were able to kill the P815 target cells, due to engagement of NKG2D, addition of mlgM or mlgM plus anti-IgM mAb did not augment this killing (data not shown).

To determine if Toso/FcR ligation could alter NK cell natural cytotoxicity against K562 cells, NK cells were incubated with K562 target cells in the absence of mlgM, the presence of mlgM, or the presence of mlgM plus anti-IgM mAb. Whereas NK cells were successful in killing the target K562 cells, neither mlgM nor mlgM plus anti-IgM mAb had an effect (Fig. 6C). Finally, as the NK cell line YTS can lyse 721.221 cells, we tested if mlgM could augment killing of 721.221 by YTS overexpressing Toso/FcR. However, mlgM treatment of the Toso/FcR-overexpressing YTS cells (cultured in cRPMI with Ig-depleted serum) did not affect killing of 721.221 cells (Fig. 6D). These findings indicate that Toso/FcR ligation does not affect the cytotoxicity of NK cells. We also tested cytokine release (TNF-α, IFN-γ) and degranulation of both NK cells and Toso/FcR-overexpressing YTS cells and found that addition of mlgM did not promote cytokine release or degranulation from either cell type (data not shown).
induced phosphorylation of ERK1/2 (Fig. 7B, center). Similar results were also obtained using NK cells freshly isolated from PBMCs and cultured in Ig-depleted serum with no IL-2 for 16 h (Fig. 7B, right). Flow cytometry of these freshly isolated cells indicates Toso/FcR is expressed and can bind exogenous IgM (data not shown). Thus, the binding of IgM to Toso/FcR generates intracellular signals indicating that Toso/FcR has the potential to regulate cellular processes.

Discussion

There is lingering controversy as to whether Toso functions to inhibit apoptosis (16) or to bind IgM (15); there is no evidence that it does both. In contrast to recently reported results (16), we provide further evidence that Toso is an IgM receptor and that its expression is not inherently antiapoptotic; hence, we agree that it might be more appropriate to refer to it as FcR (15). Little is known about Toso/FcR expression by NK cells. In this study, we show that all circulating NK cells express Toso/FcR and exposure of these cells to IL-2 leads to rapid downregulation of Toso/FcR expression that correlates with the loss of ability to bind IgM, an observation that also holds true for T cells. We analyzed the role Toso/FcR plays in activating NK cells and found that Toso/FcR, unlike the CD16 IgG receptor, does not mediate NK cell cytolytic activity; however, ligation of Toso/FcR with IgM clearly leads to the phosphorylation of PLCγ2 and ERK.

Toso expression was originally described to be important for regulating AICD due to its inhibition of apoptosis induced by TNF receptor family members in human and mouse T cells (12, 13), a viewpoint that is supported by the recent publication of Nguyen et al. (16). These reports regarding the antiapoptotic function of Toso/FcR are disputed by studies showing that Toso/FcR functions as a specific IgM receptor whose expression does not inhibit FasL-induced apoptosis. Kabugawa et al. (15) showed that Toso/FcR blocks Fas-mediated apoptosis in Jurkat T cells only when an agonistic IgM anti-Fas Ab was used, a result we confirmed (Fig. 2A, 2B) and extended to primary NK cells (Fig. 2C).

As IgM lacking anti-Fas activity is not antiapoptotic (Fig. 2B), the antiapoptotic effect of IgM anti-Fas Ab must be due to the combined binding (cross-linking) of Fas and Toso/FcR (15). As it has been shown that internalization and compartmentalization of Fas is important for apoptosis signaling (6, 11), it may be that the simultaneous binding of IgM anti-Fas Ab to Toso/FcR prevents the internalization of the trimeric Fas receptor complex or that internalization of Fas in conjunction with Toso/FcR alters the trafficking pattern of Fas such that antiapoptotic signaling is suppressed. Of potential relevance is the recent observation that Toso/FcR transports bound IgM to lysosomes for degradation (23). Alternatively, the complexing of Fas with Toso/FcR through IgM anti-Fas Ab could alter or amplify the signals generated by binding to Toso/FcR (Fig. 7) such that they now interfere with apoptosis. The fact that large excesses of nonspecific IgM do not interfere with the antiapoptotic effect of IgM anti-Fas Ab (Fig. 2B and Ref. 15) indicates that the multivalent binding of Toso/FcR and Fas conveys a large avidity advantage compared with the binding of Toso/FcR alone. Also, we acknowledge that Toso/FcR-bound non-anti-Fas IgM, under the right circumstances, could generate antiapoptotic signals. This could explain why Nguyen et al. (16) have observed that Toso/FcR expression alone can interfere with death receptor signaling, as their analyses were done in the presence of serum that likely contained IgM.
Toso/FcR signaling. (A) YTS-Toso/FcR cells were incubated with mIgM (10 μg/ml) for the indicated times. Cell lysates were immunoprecipitated with anti-Toso mAb (5 μg/ml) or control IgG2b Ab, followed by immunoblotting with anti-p-Tyr, anti-PLCγ2, or anti-Toso Abs. (B) YTS-Toso/FcR, YTS-EV, and NK cells cultured for 1 wk or freshly isolated NK cells were incubated with mIgM (5 μg/ml), anti-mIgM mAb (5 μg/ml), or mIgM plus anti-mIgM mAb for 30 min. Cell lysates were immunoprecipitated with anti-p-ERK1/2 (phospho ERK1/2) or total ERK. Bands were quantified and intensities normalized to total ERK and calibrated to values from untreated cells. Data are shown as mean ± SEM from seven independent experiments. Statistical analysis was done by two-tailed unpaired Student t test. *p < 0.05.

The expression of Toso/FcR by NK cells provides the potential greatly to expand the arsenal of receptor specificities available to NK cells for target cell recognition, much as CD16 does for ADCC. Therefore, we examined if IgM Ab reactive with target cells could mediate ADCC but found no evidence for this (Fig. 5A); moreover, we found no evidence that IgM bound to Toso/FcR can mediate redirected lysis (Fig. 5C) or potentiate natural cytotoxicity (Fig. 6). Furthermore, we found no evidence that Toso/FcR ligation affects secretion of TNF-α or IFN-γ (data not shown). However, Toso/FcR is known to be phosphorylated upon binding IgM (15). Investigation of the cytoplasmic tail of Toso/FcR indicates a number of potential signaling motifs (Eukaryotic Linear Motif resource). These include the motif DDYINV at the very end of the cytoplasmic tail, which fits criteria of both a hemITAM (24, 25) and an ITT signaling motif (26), the prevailing characteristics for both of which are Asp or Glu residues preceding the Tyr residue in the motif. Signaling downstream of NKp65, a C-lectin type receptor containing a hemITAM, increases the cytotoxicity and IFN-γ secretion of NK cells (27). ITT motifs are known to augment Grb2-mediated and PI3K signaling, and receptors with this motif can act as costimulatory receptors that are necessary for the two-signal hypothesis of lymphocyte activation (26). Of direct relevance, when this receptor is recycled or more is produced such that the presence of Toso/FcR is maintained on the surface of NK cells, at least until the NK cells are primed with IL-2. By using appropriate inhibitors, we deduce that the IL-2-mediated regulation occurs via the STAT5 signaling pathway rather than the PI3K, both of which are used in IL-2 signaling. We show that Toso/FcR expression by NK cells is remarkably sensitive to downregulation by IL-2 exposure, and this downregulation occurs regardless of the presence of IgM. Recently, Vire et al.(23) reported very rapid internalization of Toso/FcR after IgM binding in HeLa cells overexpressing the receptor and in chronic lymphocytic leukemia-derived B cells. Our signaling studies are not in contradiction to this, as there are numerous reports indicating that signaling events can continue from endocytic vesicles (see Ref. 29 for a review).

phosphorylation of PLCγ2 indicates that Toso/FcR could influence multiple signaling events in NK cells. This is consistent with reports that TLR-induced signaling in MLA-107 results in PLCγ signals that activate multiple signaling events in NK cells.

Although we are unable to detect any effector functions, IgM binding to Toso/FcR clearly is stimulatory for NK cells. Our results show that IgM binding to Toso/FcR expressed endogenously on primary NK cells and ectopically on the NK cell line YTS results in PLCγ2 and ERK phosphorylation. PLCγ2 contains a pleckstrin homology domain, an SH3 domain, and two SH2 domains, indicating the possibility for extensive interactions with PI3K and Src family kinases (see Ref. 28 for review). Thus,
an inhibitory effect, then IL-2–mediated downregulation of Toso/FcμR might help T and NK cells overcome an inhibitory threshold for activation. Clearly, the signal transduction of IgM–Toso/FcμR interaction needs to be studied in detail to address the function of Toso/FcμR in modulating the T and NK cell immune response.

Acknowledgments

We thank the National Institutes of Health Clinical Center Department of Transfusion Medicine for blood samples from healthy human donors collected under protocol 99CC-0168. We thank Seung-Chul Choi, Giovanna Peruzzi, and Linjie Tian for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References