

A Novel Inhibitory Receptor (ILT3) Expressed on Monocytes, Macrophages, and Dendritic Cells Involved in Antigen Processing

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Summary

Immunoglobulin-like transcript (ILT) 3 is a novel cell surface molecule of the immunoglobulin superfamily, which is selectively expressed by myeloid antigen presenting cells (APCs) such as monocytes, macrophages, and dendritic cells. The cytoplasmic region of ILT3 contains putative immunoreceptor tyrosine-based inhibitory motifs that suggest an inhibitory function of ILT3. Indeed, co-ligation of ILT3 to stimulatory receptors expressed by APCs results in a dramatic blunting of the increased $[Ca^{2+}]_i$ and tyrosine phosphorylation triggered by these receptors. Signal extinction involves SH2-containing protein tyrosine phosphatase 1, which is recruited by ILT3 upon cross-linking. ILT3 can also function in antigen capture and presentation. It is efficiently internalized upon cross-linking, and delivers its ligand to an intracellular compartment where it is processed and presented to T cells. Thus, ILT3 is a novel inhibitory receptor that can negatively regulate activation of APCs and can be used by APCs for antigen uptake.

Engagement of B cell antigen receptor, TCR, and several Fc receptor (FcR) complexes by cognate ligands results in tyrosine phosphorylation of cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs)¹ (1–6). Phosphorylated ITAMs recruit and activate protein tyrosine kinases, which trigger a cascade of intracellular phosphorylation events leading to cellular activation. Immune cell responses are negatively regulated when stimulatory receptors are co-ligated to inhibitory receptors. These include the low affinity FcR for IgG, Fc γ R1IB, and CD22 in B cells (7–10), the Ly-49 molecules, the killer cell inhibitory receptors (KIRs), the NKG2A/CD94 heterodimer in NK cells (11–13), and glycoprotein (gp)49B1 in mast cells and NK cells (14–16). All of these receptors are characterized by the presence of cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs; 17). Upon receptor engagement, cytoplasmic ITIMs are tyrosine phosphorylated, and subsequently bind the SH2 domain(s) of SHP-1 protein tyrosine phosphatase (18–23) and/or SHIP inositol phosphatase (24), which mediate downregulation of cell activation.

¹Abbreviations used in this paper: BCR, B cell antigen receptor; DC, dendritic cells; gp, glycoprotein; HRP, horseradish peroxidase; Ig-SF, Ig superfamily; ILT, Ig-like transcript; ILT3-HulG₁, ILT3 human IgG₁ fusion protein; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer cell inhibitory receptor; M-CSF, macrophage CSF; MFI, median fluorescence intensity.

Recently, we have described two new members of the immunoglobulin superfamily (Ig-SF), called immunoglobulin-like transcript 1 (ILT1) and ILT2 (25). These are homologous to bovine Fc γ 2 receptor (Fc γ 2R; 26), murine cell surface antigen gp49 (27, 14), human KIRs (28–30), human Fc α receptor (Fc α R; 31) and map to human chromosome 19, as do the Fc α R and the KIRs. ILT1 and ILT2 are characterized by similar extracellular regions consisting of four Ig-SF domains. Their transmembrane and cytoplasmic domains, however, differ substantially. ILT1 has a charged residue of arginine within the transmembrane region, followed by a short cytoplasmic tail with no ITAMs or ITIMs. Similar characteristics are found in human Fc α RI and bovine Fc γ 2R, which trigger cell responses via associated signal-transducing, ITAM-containing subunits. Conversely, ILT2 has a long cytoplasmic tail, which contains putative ITIMs similar to those known to bind SHP-1 in KIRs. ILT1 and ILT2 also display different tissue distributions. Although ILT1 is predominantly expressed by myeloid cells, ILT2 is expressed by B cell lines. Thus, ILT1 and ILT2 may mediate activation of myeloid cell responses and inhibition of B cell activation, respectively.

Here we characterize a third member of the ILT family, called ILT3, which, similarly to ILT2, displays a long cytoplasmic tail containing putative ITIMs. By using an anti-ILT3 mAb, we have found that ILT3 is selectively ex-

pressed on myeloid APCs. Functional studies show that ILT3 behaves as an inhibitory receptor when cross-linked to a stimulatory receptor. A cytoplasmic component of the ILT3-mediated negative signaling pathway is the SH2-containing phosphatase SHP-1, which becomes associated with ILT3 upon cross-linking. Furthermore, ILT3 is internalized and ILT3 ligands are efficiently presented to specific T cells, suggesting that ILT3 may be involved in antigen uptake and presentation.

Materials and Methods

Cells. THP1 is a human myeloid cell line. C1R and 721.221 are MHC class I-deficient, EBV-transformed human B cell lines. Jurkat is a human T cell line. All of these cells were grown in RPMI, 10% FCS. Monocytes were prepared from PBMCs by adhesion to plastic. In brief, PBMCs were isolated from whole blood by Ficoll-Hypaque density gradient, washed with RPMI, resuspended at 5×10^6 /ml in RPMI, 10% FCS and cultured in six-well plates for 1 h at 37°C. Nonadherent cells were removed by washing twice with RPMI. Adherent cells were cultured overnight in RPMI, 10% FCS supplemented with 50 ng/ml GM-CSF and 1,000 U/ml IL-4. Dendritic cells (DCs) were obtained from monocytes as previously described (32). Macrophages were obtained by culturing monocytes for 10 d in six-well plates at a concentration of 10^6 cells/ml in RPMI, 20% human serum supplemented with 1 ng/ml macrophage CSF (M-CSF) (33, 34).

cDNA Synthesis, Oligonucleotide Primers, PCR, and Cloning. The 3' end of ILT3 was cloned by rapid amplification of cDNA 3' end (35). In brief, RNA was prepared from 721.221 and C1R cells as described (36) and single-strand cDNA was synthesized by reverse transcription of 1 µg of total RNA using Moloney murine leukemia virus reverse transcriptase and a (dT)₁₇ adaptor in a reaction volume of 20 µl. cDNA was amplified by two nested PCRs. The 5' oligonucleotides were CCCCAGCGACC-CCCTGGA (nucleotide 926–944 of ILT2) and ACGGTGGC-CTCAGGAGAGA (nucleotide 1003–1021 of ILT2) (25). In both PCRs, the 3' primer corresponded to the adaptor primer. PCRs (50 µl volume) contained 1 µl of total cDNA sample, PCR primers (0.5 µM each), 2'-deoxynucleoside 5'-triphosphates (125 µM each), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatin, and 0.25 units of Taq polymerase. PCR was carried out for 35 cycles, each consisting of 1-min steps at 94, 55, and 72°C. Amplified products were gel purified in 1.2% agarose gel, cloned into pCRII (Invitrogen, San Diego, CA), and sequenced. The 5' end of ILT3 was cloned by two consecutive cycles of 5' rapid amplification of cDNA ends (35).

Production of ILT3 Human IgG₁ Fusion Protein. To produce ILT3 as a soluble fusion protein, we constructed a chimeric gene consisting of ILT3 extracellular domains and human IgG1 constant regions. The cDNA fragment encoding the ILT3 extracellular region was amplified by PCR from cloned plasmid DNA. The 5' primer (GATCGAATTCATGATCCCCACCTTCACGGCT) contained an EcoRI restriction site (*italic*) and the ILT3 start codon. The 3' primer (CTGATCAAGCTTATACTTACCCT-CCCAGTGCCTTCTCAGACC) provided a HindIII restriction site (*italic*), a splice donor sequence, and seven ILT3 codons preceding the transmembrane domain. The ~800-bp PCR product was cut with EcoRI and HindIII, and ligated into an expression vector containing the exons for hinge, CH2 and CH3 region of human IgG₁, the guanosine phosphotransferase gene conferring

resistance to mycophenolic acid, and the κ promoter for the expression in mouse myeloma (37). Transfections, selection of secreting transfectants, and purification of fusion protein from culture supernatants were performed as described (38).

Production of anti-ILT3 mAbs. 10-wk-old, female BALB/c mice (Iffa-Credo, L'Arbresle, France) received an initial injection of 100 µg of ILT3 human IgG₁ fusion protein (ILT3-HuIgG₁), mixed 1:1 (vol/vol) with Alu-Gel-S (Serva Biochemicals, Paramus, NJ), behind the neck. 4 wk later, they were given a booster immunization with the same immunogen, followed after 2 wk by a final injection of 100 µg of purified ILT3-HuIgG₁. 3 d later, mice were killed and draining lymph node cells were isolated and fused with the myeloma fusion partner, Ag8.653, using polyethylene glycol 4000. Hybridoma supernatants were screened in two steps. First, an ELISA was performed using ILT3-HuIgG₁ in the coating step and human-adsorbed alkaline phosphatase-labeled goat anti-mouse IgG as secondary antibody. Supernatants from clones that were positive in ELISA were then tested by FACS[®] analysis for staining C1R cells by flow cytometry.

Transfections. ILT1, ILT2, and ILT3 cDNAs were subcloned into pCDNA3 (Invitrogen) and transfected into COS7 cells by lipofectin (GIBCO BRL, Gaithersburg, MD). Cell surface expression of ILTs was assessed 48 h after transfection by FACS[®] analysis. A stable transfectant of ILT3 in Jurkat T cells was obtained as previously described (28).

Antibodies and FACS[®] Staining. Before staining, all the cells were preincubated with PBS, 20% human serum for 30 min on ice, to block Fc receptors. In two- and three-color staining experiments, cells were stained with the following primary mAbs: OKT3 (anti-CD3, IgG2a; American Type Culture Collection, Rockville, MD), CD16 (IgG2a; Milan Analytica AG, La Roche, Switzerland), 3C10 (anti-CD14, IgG2b; American Type Culture Collection), 1F5.4 (anti-CD20, IgG2a; American Type Culture Collection), M-T102 (anti-CD1a, IgG2b; PharMingen, San Diego, CA), L243 (anti-HLA-DR, IgG2a; American Type Culture Collection), and HB15a (anti-CD83, IgG2b; provided by Dr. T. Tedder, Duke University, Durham, NC). As secondary antibodies, we used human-adsorbed FITC- or PE-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b (Southern Biotechnology Assoc., Inc., Birmingham, AL). In three-color stainings, biotin-labeled goat anti-mouse IgG2a was followed by streptavidin-allophycocyanin (SBA). Stained cells were analyzed by flow cytometry on a FACStar[®] Plus (Becton Dickinson, Mountain View, CA) using the LYSYS II software.

Immunoprecipitations. Cells were surface labeled with 1 mCi of Na¹²⁵I using the sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate method (39). For metabolic labeling with [³²P]orthophosphate, cells were incubated in phosphate-free DMEM (Sigma Chemical Co., St. Louis, MO) containing 10% Tris-buffered saline-dialyzed FCS for 1 h. [³²P]orthophosphate (Amersham Corp., Arlington Heights, IL) was then added at 0.5 mCi/ml and incubation was continued for 5 h. After surface or metabolic labeling, cells were lysed in 1% Triton X-100, 100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. After overnight preclearing with protein G-sepharose, lysates were incubated with culture supernatants of either ZM3.8 (anti-ILT3, IgG₁) or control IgG1 mAbs (5.133, anti-NKAT4 [40] and 1B7.11, anti-2,4,6 TNP, American Type Culture Collection) at +4°C for 4 h, and immune complexes were precipitated by addition of protein G-sepharose for 1 h at +4°C. Precipitates were washed 3 times with lysis buffer, followed by a final wash with 10 mM Tris-HCl, pH 7.4, and resuspended in nonreducing or reducing sample buffer. SDS-PAGE analysis was performed according to a standard procedure. After

the run, gels were dried and exposed to autoradiography film (Amersham Corp.) for 2–5 d.

Measurement of Cytosolic Calcium in Macrophages and Monocytes. Monocytes and macrophages were loaded with Indo-1 AM (Sigma Chemical Co.) as described (41). In stimulation experiments, anti-CD11b mAb (44aabc; American Type Culture Collection) and anti-CD16 mAb (B73.1; American Type Culture Collection) were added to monocytes and macrophages, respectively, followed by a F(ab')₂ goat anti-mouse IgG (Milan Analytica) as cross-linker. Cells were then analyzed on a flow cytometer (FACS® Vantage or Coulter Elite Enhanced Sort Performance [Coulter Corp., Hialeah, FL]) to detect Ca²⁺ fluxes. Stimulation of monocytes with the anti-HLA-DR mAb 3.8B1 (Cella, M., and A. Lanzavecchia unpublished data) was performed without a cross-linker. In inhibition experiments, cells were preincubated for 10 min at room temperature either with ZM3.8 mAb, or with the anti-MHC class I W6/32 mAb. After washing with PBS, either anti-CD11b, anti-HLA-DR, or anti-CD16 mAbs were added followed by the cross-linking antibody, and cells were analyzed by flow cytometry to detect Ca²⁺ fluxes. Only live (based on forward scatter criteria) and Indo-1-loaded cells (based on 405 nm versus 525 nm emission spectra) were included in the analysis.

Immunoblotting. For antiphosphotyrosine blots, monocytes (2 × 10⁶) were incubated for 2 min at 37°C with medium, ZM3.8 mAb, 3.8B1 mAb, or with both mAbs in the absence or in the presence of a secondary cross-linker. Cells were washed in ice-cold PBS and lysed as previously described (20). Cell lysates were boiled, separated on SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase (HRP)-coupled 4G10 (500 ng/ml; Upstate Biotechnology Inc., Lake Placid, NY). Immunoblotted proteins were visualized by chemiluminescence using the enhanced chemiluminescence detection reagents (Amersham Corp.). For anti-SHP blots, 10⁷ monocytes were plated on six-well tissue culture plates previously coated with purified ZM3.8 or 5.133 mAbs at 50 μg/ml, centrifuged for 2 min, shifted to 37°C for 2 min, collected in cold PBS, 0.5 mM EDTA, and washed twice. Identical number of cells stimulated with ZM3.8 or 5.133 mAbs were lysed and immunoprecipitated with ZM3.8 and protein G as described above. Precipitated samples were boiled for 5 min, separated by SDS-PAGE, and transferred

to nitrocellulose (Hybond-C extra; Amersham Corp.). Membranes were blocked with PBS, 3% nonfat, dry milk and probed anti-SHP-1 rabbit antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) followed by HRP-labeled goat anti-rabbit Ig (SBA). Immunoblotted proteins were visualized by chemiluminescence. Membranes were stripped of antibody by incubating for 30 min at 50°C in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, according to the protocol supplied by Amersham Corp. Stripped blots were reprobed after blocking with anti-SHP-2 rabbit antiserum (Santa Cruz Biotechnology).

Internalization Assay. Monocytes were cultured for 24–48 h in culture medium containing GM-CSF and IL-4, as described above. One aliquot of monocytes was fixed for 1 min at room temperature in RPMI, 0.05% glutaraldehyde. Another aliquot was used without fixation. Both samples were stained with ZM3.8 mAb for 40 min on ice, washed twice with ice-cold PBS, 1% FCS, and subsequently stained with biotin-labeled F(ab')₂ goat anti-mouse IgG for 1 h on ice. After washing with ice-cold PBS, 1% FCS, cells were incubated at 37°C in prewarmed RPMI, 10% FCS for various times to allow internalization. Cells were then cooled on ice, washed once with PBS, 0.1% Na₃, stained with PE-conjugated streptavidin for 30 min on ice, washed, and analyzed by FACS®. As a measure of internalization in nonfixed cells, we used the percent decrease of the median fluorescence intensity (MFI) as compared to control samples kept at 4°C. The percentage decrease of MFI observed in fixed cells was taken as a measure of the off-rate of the antibody at 37°C.

Antigen Presentation Assay. 4 × 10⁴ irradiated monocytes (3,000 rad) were co-cultured with 8 × 10⁴ cells of the B13 T cell clone (42) in 96-well flat-bottom microplates in the presence of serial dilutions of IgG1 mAbs. mAbs used in the assay were the following: ZM3.8 (anti-ILT3, IgG₁) or 5.133 (anti-NKAT4 KIR, IgG₁), 1B7.11 (anti-2,4,6 TNP, IgG₁; American Type Culture Collection), and 32.2 (anti-FcγRI, IgG₁; American Type Culture Collection). After 48 h, the cultures were pulsed with [³H]thymidine (1 μCi/well, specific activity 5 Ci/mmol), and the radioactivity incorporated was measured after additional 16 h. The data were plotted against the concentration of mAbs determined by ELISA using a purified mouse IgG₁ (anti-CD68, Dako Corp., Carpinteria, CA) as a standard.

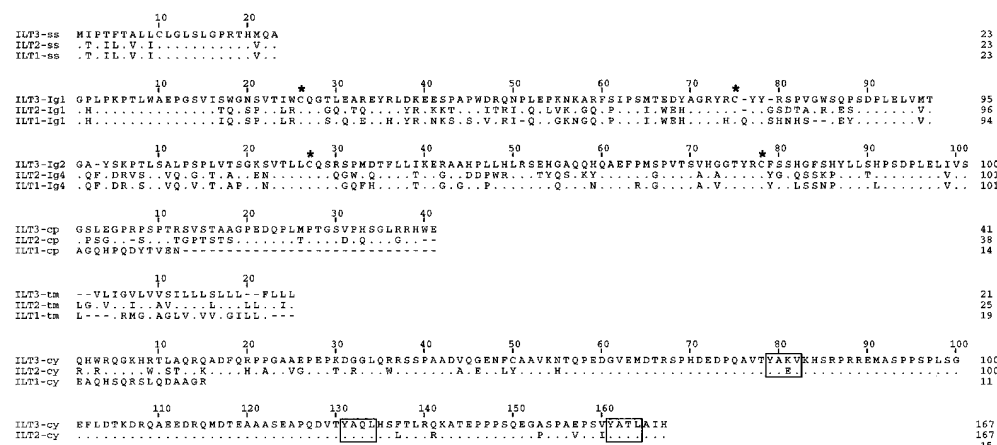


Figure 1. Amino acid sequence of human ILT3, aligned with ILT1 and ILT2. Since ILT1 and ILT2 consist of four Ig-SF domains, only the two domains with higher homology to ILT3 were included in the alignment. The alignment was generated by the Clustal method. Gaps (*dashes*) were introduced to maximize homologies. Amino acids identical to the consensus are indicated by dots. Conserved cysteines involved in disulfide bonds in the extracellular domains are identified by asterisks. Cytoplasmic tyrosine-based motifs potentially involved in signal transduction and/or endocytosis are boxed.

ILT3 has no N-linked glycosylation sites (N-X-S/T). Amino acid residues are numbered on the right side, beginning with the first residue of each of the predicted domains. *ss*, signal sequence; *Ig1–4*, Ig-SF extracellular domains; *cp*, connecting peptide; *tm*, transmembrane domain; *cy*, cytoplasmic domain. ILT3 cDNA sequence is available from EMBL/GenBank/DBJ under accession number U82979.

Results

ILT3 Is a Member of the ILT Multigene Family Located on Human Chromosome 19. During the process of cloning the 3' end of ILT2 cDNA from EBV-B cell lines (25), we obtained an additional cDNA sequence that differed from ILT2 by several amino acid residues. Amplification of the 5' end of this fragment yielded a new cDNA, called ILT3, which contains a single open reading frame encoding a transmembrane protein of 447 amino acids with a predicted molecular mass of ~ 47 kD (Fig. 1). The amino acid sequence begins with a hydrophobic signal peptide of 23 amino acids followed by an extracellular region composed of two C2 type Ig-SF domains (43). Each domain shows two characteristic cysteines that are 49 and 50 residues apart, flanked by conserved residues (Val-x-Leu/Ile-x-Cys and His/Tyr-x-Gly-x-Tyr-x-Cys-Tyr/Phe, respectively, where x is any amino acid). The putative transmembrane domain of ILT3 consists of 21 amino acids, followed by a long cytoplasmic region of 167 amino acids, which is characterized by the presence of one Tyr-x-x-Val motif followed by two Tyr-x-x-Leu motifs spaced by 26 amino acid residues. These Tyr-x-x-Leu pairs and their spacing are reminiscent of the Tyr-x-x-Leu motifs identified in KIRs

as binding sites for protein tyrosine phosphatase SHP-1 (19–23).

Comparison of the predicted amino acid sequence of ILT3 with Ig-SF protein sequences, revealed that ILT3 is closely related to ILTs (64% similarity with ILT2), bovine Fc γ 2R (45%), murine cell surface antigen gp49 isoforms (39–43%), and, to a lesser extent, to KIRs (23–32%) and human Fc α R (28%). Genomic DNA analysis of human-hamster hybrid cell lines, each with a different partial complement of human chromosomes, showed hybridizing bands only in samples containing human chromosome 19 (data not shown). Thus, ILT3 belongs to the ILT family which maps to human chromosome 19, as do the Fc α R and the KIR genes. Expression of ILT3 by RNA blot analysis revealed a major transcript of ~ 1.6 kb (data not shown). Interestingly, this transcript was weakly expressed in the B cell lines C1R or 721.221, from which ILT3 was initially cloned, whereas it was predominantly expressed in monocytes purified from PBMCs and in the myeloid cell line THP1. No transcripts were detected in the T cell line Jurkat.

ILT3 Is Expressed on Monocytes, Macrophages, and Dendritic Cells. To characterize the ILT3-encoded cell surface mol-

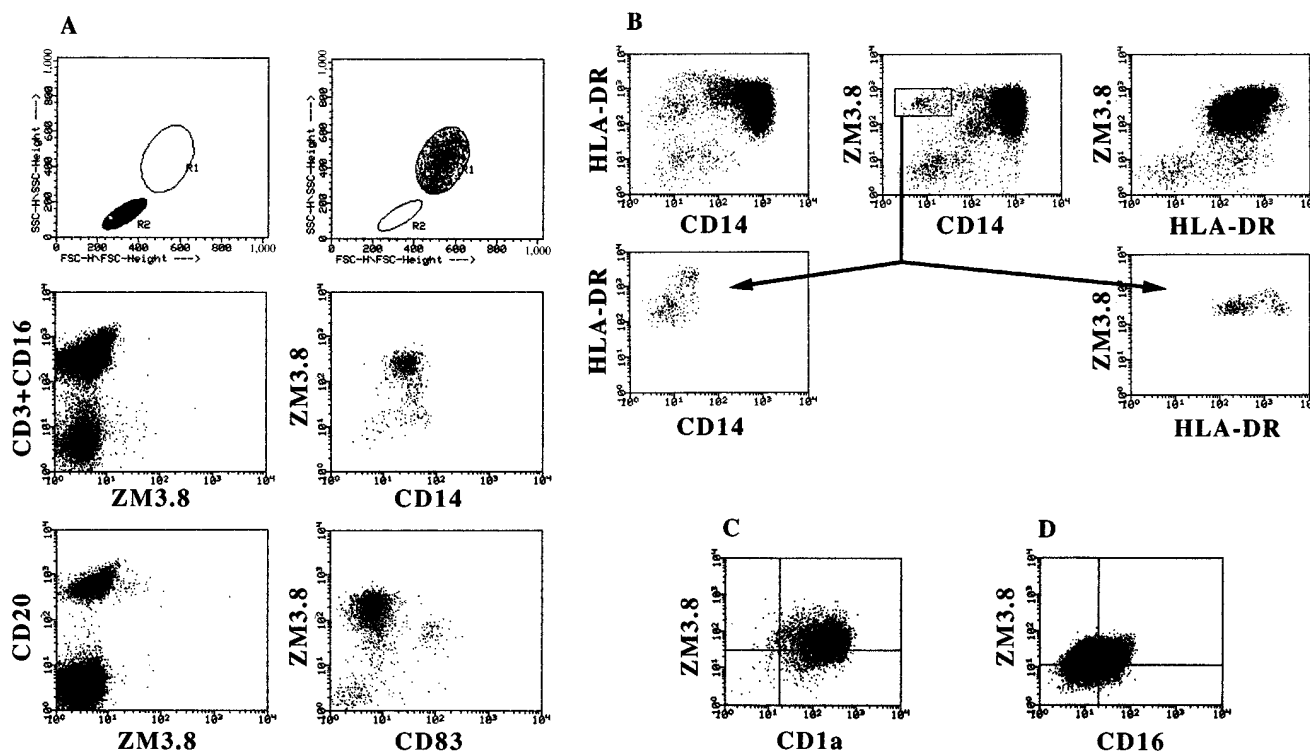


Figure 2. Expression of ILT3 in monocytes, primary DCs, monocyte-derived DCs, and macrophages. (A) mAb ZM3.8 stains CD14⁺ monocytes in PBMC and CD83⁺ DCs in a monocyte-enriched population (*right*). On the contrary, CD3⁺ T cells, CD16⁺ NK cells, or CD20⁺ B cells are not stained (*left*). PBMCs and monocyte-enriched populations were subjected to two-color staining. Lymphocytes (*left*) and monocytes (*right*) were gated using forward and side scatter (FSC and SSC) parameters. (B) ILT3 is expressed on CD14⁺ monocytes and on a subset of CD14⁻/HLA-DR^{high} cells, which corresponds to circulating primary DCs (46). Monocytes were enriched from peripheral blood and subjected to three-color staining using anti-CD14 (FITC), ZM3.8 (PE), and anti-HLA-DR (APC). (C) Monocyte-derived DCs express both CD1a and ILT3. (D) Macrophages express low levels of both CD16 and ILT3. DCs and macrophages were derived from monocytes under appropriate culture conditions and subjected to two-color staining. Negative controls were located in the lower left quadrants.

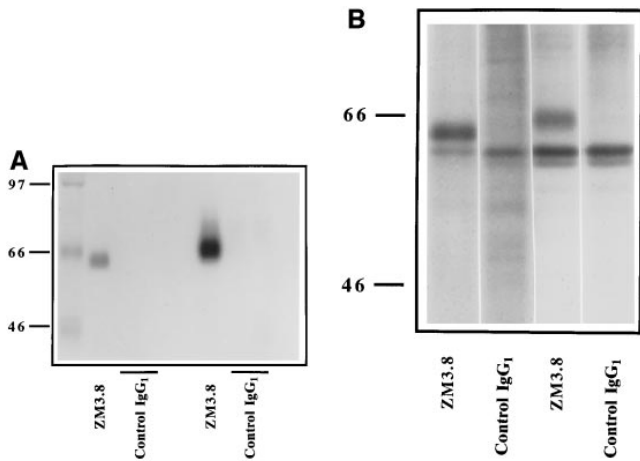


Figure 3. (A) SDS-PAGE analysis of ILT3 immunoprecipitated from ¹²⁵I-labeled, ILT3-transfected Jurkat T cells and monocytes. ILT3 appears as a ~55-kD band in ILT3-transfected cells and as a ~58–60-kD band in monocytes. (B) When ILT3 is precipitated from ³²P-labeled cells, it is detectable as a constitutively phosphorylated molecule.

ecule, we produced anti-ILT3 mAbs using a soluble ILT3-HuIgG₁ fusion protein as an immunogen. The ZM3.8 mAb specifically recognized ILT3 on transiently transfected COS cells (data not shown). In PBMCs, ZM3.8 mAb stained only CD14⁺ monocytes, whereas no staining was observed in CD3⁺ T cells, CD16⁺ NK cells, or CD20⁺ B cells (Fig. 2 A). Interestingly, in monocyte-enriched populations derived from peripheral blood, ZM3.8 also stained CD83⁺ cells (Fig. 2 A), as well as CD14⁻/HLA-DR^{high} cells (Fig. 2 B). Both of these cell populations correspond to circulating primary DCs (44–46). In addition, ILT3 was expressed by both DCs and macrophages derived from monocytes cultured with GM-CSF plus IL-4 or M-CSF, respectively (Fig. 2, C and D; 32–34).

We also tested ILT3 expression in several cell lines. Among four myelo-monocytic cell lines (U937, Monomac6, THP1, and HL-60), only THP1 reacted with ZM3.8. All tested EBV-B cell lines ($n = 10$) were negative, with the exception of C1R. No cell surface expression was detected either on purified fresh B cells or on B cells activated by CD40L in the presence of IL-4, IL-2, and IL-10 (data not shown). Finally, ZM3.8 did not stain neutrophils (data not shown). Thus, ILT3 is selectively expressed on myeloid APCs.

To define the biochemical characteristics of ILT3, we carried out immunoprecipitations from ILT3-transfected Jurkat T cells and monocytes (Fig. 3). A prominent band of ~55 kD in ILT3-transfected T cells was detected under nonreducing and reducing conditions, while a ~58–60 kD band was detected in monocytes (Fig. 3 A) and in THP1 (data not shown). *N*- and *O*-deglycosylation of immunoprecipitates did not produce any change in the size of the bands. After labeling cells with [³²P]orthophosphate, ILT3 was immunoprecipitated as a constitutively phosphorylated molecule (Fig. 3 B). The discrepancy in apparent molecular mass of ILT3 between ILT3-transfected T cells and mono-

cytes may be due to a different degree of phosphorylation in distinct cell types.

Intracellular Ca²⁺ Mobilization Responses Triggered by CD11b, MHC Class II, and CD16 in APCs Are Negatively Regulated by Co-ligation with ILT3. The presence of putative ITIMs suggested an inhibitory role of ILT3 on APC activation. To test this hypothesis, we triggered monocytes and macrophages with mAbs that have been shown to induce Ca²⁺ mobilization, such as anti-CD11b (47, 48), anti-HLA-DR (49–51), and anti-FcγRIII (CD16; 52) mAbs, and tested whether recruitment of ILT3 to the triggering receptors inhibits Ca²⁺ mobilization. As shown in Fig. 4 A, ligation of surface CD11b with a specific mAb followed by a secondary cross-linking antibody elicited a rapid and transient rise in intracellular calcium concentration ([Ca²⁺]_i). On the contrary, preincubation of monocytes with ZM3.8 mAb, followed by addition of anti-CD11b and co-cross-linking of CD11b and ILT3 by a secondary antibody, resulted in complete inhibition of the [Ca²⁺]_i increase (Fig. 4 D). In control experiments, the [Ca²⁺]_i increase induced by CD11b was not affected by co-ligation of CD11b with MHC class I. Also, cross-linking of ILT3 alone did not evoke Ca²⁺ mobilization, indicating that ILT3 is not a stimulatory receptor (data not shown).

It has been reported that some anti-MHC class II mAbs can trigger functional responses in B lymphocytes (49–51). We have recently developed an anti-HLA-DR mAb, called 3.8B1, which triggers a rapid rise in [Ca²⁺]_i, even in the absence of a secondary cross-linking antibody (Fig. 4 B). Co-ligation of ILT3 and HLA-DR resulted in significant inhibition of [Ca²⁺]_i increase (Fig. 4 E). Interestingly, HLA-DR-mediated stimulation of monocytes was not affected by preincubation of monocytes with anti-ILT3 anti-

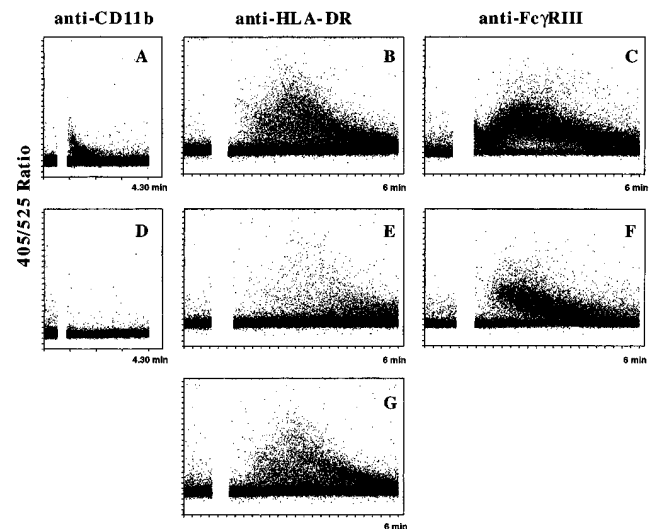


Figure 4. Intracellular Ca²⁺ mobilization induced in monocytes and macrophages via CD11b (A), HLA-DR (B), and FcγRIII (C) are inhibited upon cross-linking with ILT3 (D–F). G shows that in the absence of a cross-linking antibody, ILT3 does not inhibit Ca²⁺ flux triggered by the 3.8B1 anti-HLA-DR mAb.

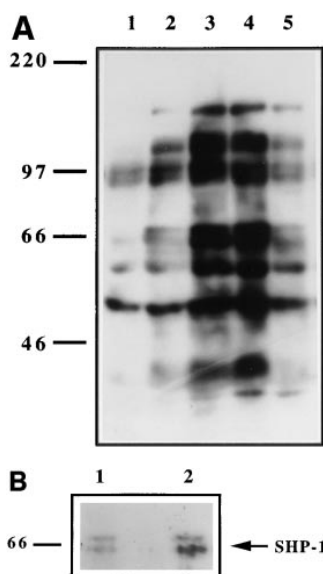


Figure 5. (A) Protein tyrosine phosphorylation stimulated by treatment of monocytes with the anti-HLA-DR mAb 3.8B1 is inhibited by co-ligation of HLA-DR with ILT3. Monocytes were incubated with medium alone (lane 1), with ZM3.8 mAb (anti-ILT3; lane 2), with 3.8B1 mAb (anti-HLA-DR; lane 3) or with both mAbs in the absence (lane 4) or in the presence (lane 5) of a secondary cross-linking antibody. Cell lysates were separated on SDS-PAGE, transferred to nitrocellulose, and probed with HRP-coupled antiphosphotyrosine mAb 4G10. (B) Association of SHP-1 with ILT3 is increased upon ILT3 cross-linking. Monocytes were stimulated with ZM3.8 (lane 2) or with a control IgG (5.133 mAb) (lane 1) coated on plastic

plates. Cells were kept at 37°C for 2 min, harvested, and lysed. ILT3 was immunoprecipitated with ZM3.8. Proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with anti-SHP-1, followed by HRP-conjugated goat anti-rabbit Ig. The migration of SHP-1 is marked by the arrow.

body in the absence of the secondary cross-linking antibody, indicating that recruitment of ILT3 to the stimulatory receptor is necessary to block the $[Ca^{2+}]_i$ increase (Fig. 4 G).

The low affinity receptor for IgG, Fc γ RIII, is expressed on NK cells and macrophages and has been shown to trigger Ca^{2+} mobilization in NK cells (52, 53). Similarly, we showed that cross-linking of Fc γ RIII in macrophages evokes a strong increase of $[Ca^{2+}]_i$ (Fig. 4 C). Despite the low expression of ILT3 on macrophages (see Fig. 2 D), co-ligation of ILT3 and Fc γ RIII inhibited the $[Ca^{2+}]_i$ increase, especially at early time points (Fig. 4 F). Together, these results demonstrate that co-ligation of ILT3 with a triggering receptor results in signal extinction.

SHP-1 Is Recruited to ILT3 upon Cross-linking. To test whether inhibition of Ca^{2+} mobilization responses was paralleled by inhibition of tyrosine phosphorylation, we performed antiphosphotyrosine immunoblotting on whole cell lysates of monocytes stimulated via HLA-DR in the absence or in the presence of co-ligation with ILT3. As shown in Fig. 5 A, monocytes triggered via HLA-DR displayed a substantial increase of tyrosine phosphorylation (lane 3), as compared to monocytes incubated with medium alone (lane 1) or with anti-ILT3 mAb (lane 2). Co-ligation of ILT3 with HLA-DR resulted in a dramatic reduction of the intensity and number of tyrosine phosphorylated species (lane 5). In the absence of secondary cross-linking antibody, ILT3 did not block HLA-DR-triggered tyrosine phosphorylation increase (lane 4).

Since in NK cells, KIRs downregulate calcium mobilization and tyrosine phosphorylation by recruiting SHP-1 and SHP-2 (19–23), we tested whether these phosphatases could be also involved in the negative signaling pathway

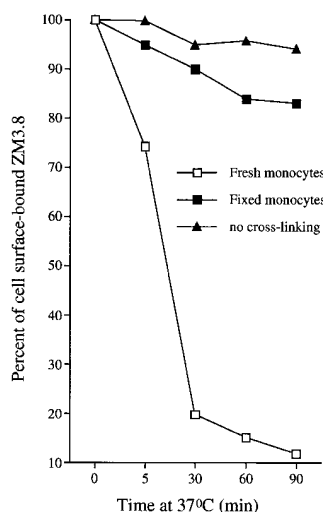


Figure 6. ZM3.8 mAb is internalized upon cross-linking. ZM3.8 bound to monocytes on ice did not disappear from the cell surface when the cells were shifted at 37°C, in the absence of a cross-linking antibody (▲). After cross-linking with a F(ab')₂ biotin-labeled secondary antibody, ZM3.8 was rapidly internalized, becoming undetectable by FACS analysis in 30–60 min (□). In fixed cells, only a minimal decrease of the median fluorescence intensity was detected over time (■). Cell surface-bound ZM3.8 was revealed by PE-conjugated goat anti-mouse IgG1 (▲) or by PE-conjugated streptavidin (□, ■). The MFI was determined by FACS analysis. The percentage decrease of MFI as compared to a control sample kept at 4°C was used as a measure of internalization.

mediated by ILT3. ILT3 was cross-linked on monocytes by ZM3.8 mAb attached to the plastic surface of tissue culture plates and subsequently immunoprecipitated from cell lysates. In control experiments, ILT3 was immunoprecipitated from unstimulated cells. Immunoblotting of immunoprecipitates with anti-SHP-1 antibodies demonstrated association of SHP-1 with ILT3 that significantly increases upon ILT3 cross-linking (Fig. 5 B). On the contrary, no association with SHP-2 was detected (data not shown). These results implicate SHP-1 as a cytosolic component of the signal extinction mediated by ILT3.

Cross-linking of ILT3 on Monocytes Results in Receptor Internalization and Delivery into an Antigen-processing Compartment. Since ILT3 is selectively expressed on APCs and displays putative tyrosine-x-x-valine/leucine internalization motifs in the cytoplasmic tail (54–56), we analyzed the ability of ILT3 to endocytose and deliver its ligand to an antigen-processing and loading compartment. As shown in Fig. 6, ZM3.8 mAb bound to monocytes was not internalized when the cells were shifted to 37°C. We therefore examined whether internalization occurs in the presence of a secondary cross-linker. ZM3.8 mAb was bound on ice to monocytes and cross-linked by a biotinylated F(ab')₂ secondary antibody. Cells were subsequently warmed to 37°C to allow internalization. After various time points, cells were returned to ice and the amount of ZM3.8 mAb remaining on the cell surface was determined by flow cytometry using PE-conjugated streptavidin. Freshly isolated monocytes efficiently internalized bound ZM3.8 mAb, as demonstrated by a 60–80% reduction after 30 min at 37°C and by a complete disappearance after 60–90 min (Fig. 6). In control experiments, no significant decrease of surface-bound ZM3.8 was observed using fixed monocytes, ruling out a detachment of the primary or secondary antibodies from the cell surface at 37°C (Fig. 6). In addi-

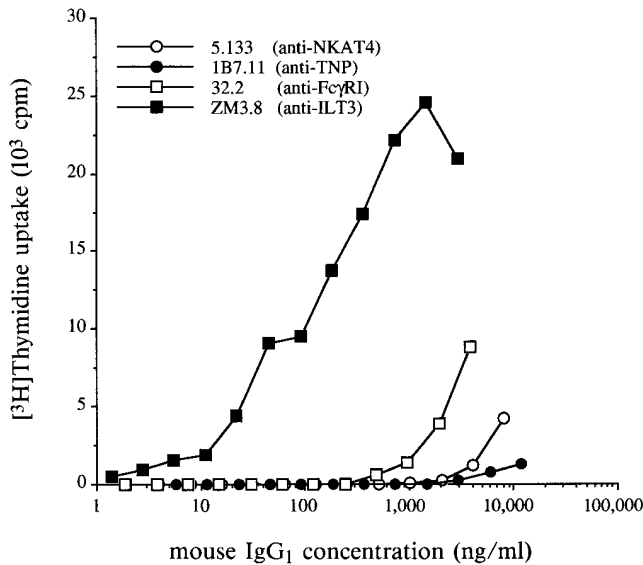


Figure 7. Presentation of ZM3.8 mAb to a T cell clone specific for mouse IgG1 by irradiated monocytes. ZM3.8 mAb (■) is presented 50–100-fold more efficiently than anti-FcγRI (□) and 400–500-fold more efficiently than anti-TNP (●) and anti-NKAT4 KIR (○) mAbs, which do not stain monocytes.

tion, when ¹²⁵I-labeled streptavidin was added as a third step reagent before incubation at 37°C, no significant decrease of cell-bound radioactivity was observed over time, thus excluding shedding of ILT3 from the cell surface (data not shown).

To further investigate a possible role of ILT3 in antigen capture, we evaluated the ability of monocytes to present ZM3.8 mAb to a CD4⁺ class II-restricted T cell clone specific for a mouse IgG1 peptide epitope (42). The presentation of the ZM3.8 mAb was compared to that of IgG1 mAbs that bind to other receptors (anti-FcγRI mAb) or do not bind to surface molecules and are taken up in the fluid phase [anti-TNP and anti-NKAT4 KIR mAbs]. As shown in Fig. 7, monocytes presented ZM3.8 mAb to T cells 50–100-fold more efficiently than the anti-FcγRI mAb, and 400–500-fold more efficiently than anti-TNP and anti-NKAT4 KIR mAbs. Together, these experiments indicate that ILT3 can efficiently deliver its ligand to an intracellular compartment where class II loading can occur.

Discussion

Previous work has extensively shown that inhibitory receptors regulate cell activation in B, NK, mast cells, and subsets of T cells when co-ligated with ITAM-containing

stimulatory receptor complexes. Our data extend this concept to professional APCs, showing that ILT3 negatively regulates APC functional responses triggered via stimulatory receptors, such as CD11b, CD16, and even MHC class II. In addition to its inhibitory function, ILT3 is involved in antigen uptake. It is noteworthy that ILT3 is expressed on DCs. These are a unique type of leukocytes whose primary function is to efficiently capture antigens, process them, and present them to naive T cells (57). Although the uptake and processing of antigen is a major function of DCs, only a few mechanisms and receptors specialized for antigen capture have been identified on DCs (58–60). Our data indicate that ILT3 is a unique receptor expressed in DCs, able to target the ligand into processing and peptide-loading compartments. Thus, ILT3 displays a dual function of inhibitory receptor and antigen-capturing molecule. A similar dual function has been previously shown for the low affinity receptor for IgG, FcγRIIB (7, 8).

The ligand of ILT3 is still unknown. ILT3 is closely related to bovine Fcγ2R and, to a lesser extent, to human FcαR, suggesting that ILT3 may be an Fc receptor. However, we did not detect any significant binding of human monomeric and heat-aggregated IgM, IgG, IgA, and IgE to ILT3-transfected Jurkat T cells (data not shown). ILT3 also shows homology to KIRs, which suggests that it may be a receptor for MHC class I molecules. We have investigated this possibility by analyzing binding of soluble ILT3-HuIgG₁ fusion protein to MHC class I transfectants in the class I-deficient mutant 721.221. No significant binding could be detected by FACS[®] analysis under the same experimental conditions in which KIR-HuIgG fusion proteins bind to MHC class I molecules (data not shown). We are investigating the possibility that ILT3 is a receptor for cell surface molecules related to MHC class I molecules, such as nonclassical class I molecules (CD1 [61], MR1 [62], MIC [63]), for MHC class II molecules, or for serum factors such as complement components.

ILT3 is a member of the ILT subfamily of Ig-SF molecules, which is located on chromosome 19, most likely in close linkage with KIRs. Another member of this family, ILT2, is characterized by similar cytoplasmic tyrosine-based motifs such as ILT3, and is expressed in B cell lines, suggesting that this may be a putative inhibitory receptor in B cells. We have also cloned additional cDNAs which encode novel molecules homologous to ILT2 and ILT3 (ILT4 and ILT5, GenBank accession numbers AF000574 and AF000575). Thus, the number of Ig-SF inhibitory receptors with different tissue distribution and specificity is likely to increase.

We thank S. Bahram, K. Campbell, and R. Torres for critical reading of the manuscript.

The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche Ltd., Basel, Switzerland.

Received for publication 30 January 1997.

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