Cutting Edge: Human Myelomonocytic Cells Express an Inhibitory Receptor for Classical and Nonclassical MHC Class I Molecules[1]

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Leukocyte activation can be negatively regulated by inhibitory receptors specific for MHC class I molecules. While one inhibitory receptor, Ig-like transcript 2 (ILT2), is expressed by all lymphoid and myelomonocytic cell types, other receptors display a more selective tissue distribution. Here we characterize an inhibitory receptor, termed ILT4, which is selectively expressed in monocytes, macrophages, and dendritic cells (DCs), binds classical class I molecules and the nonclassical class I molecules HLA-G, and transduces negative signals that can inhibit early signaling events triggered by stimulatory receptors. ILT4 may control inflammatory responses and cytotoxicity mediated by myelomonocytic cells and may modulate their Ag-presenting functions, focusing immune responses to microbial challenges and avoiding autoreactivity. ***The Journal of Immunology,*** **1998, 160: 3096–3100.**

Natural killer (NK) cells display cell surface receptors that monitor expression of class I molecules on neighboring cells and deliver a negative signal that blocks NK cell-mediated cytotoxicity of class I-bearing normal cells (1). Inhibitory MHC class I receptors belong to either the Ig-superfamily (Ig-SF),[3] named killer cell inhibitory receptors, or to the C-type lectin superfamily (2, 3). Both types of receptors display cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Upon receptor engagement with class I molecules, ITIMs are tyrosine phosphorylated and recruit protein tyrosine phosphatases, which appear to be the major cytosolic mediators of NK cell inhibition (4).

Recently, new families of Ig-SF receptors have been identified that are homologous to killer cell inhibitory receptors in their extracellular domains and display cytoplasmic ITIMs (reviewed in Ref. 5). Interestingly, these receptors are not expressed just in NK cells but also in other cell types involved in the immune response. One of these receptors, Ig-like transcript (ILT) 2, is expressed on NK and T cell subsets, B lymphocytes, and myelomonocytic cells and is specific for MHC class I molecules (6–8). Other receptors show a more restricted pattern of expression. ILT3 is expressed only in myelomonocytic cells and inhibits cell activation, but its ligand specificity is unknown (9). ILT4 and ILT5 have also been cloned from cDNA derived from myelomonocytic cells and are predicted to encode glycoproteins characterized by an extracellular region of 4 Ig-SF domains and a cytoplasmic tail containing 3 or 4 ITIMs, respectively (8, 9). However, no information is available on their ligands and their function in myelomonocytic cells.

Here we show that ILT4 is selectively expressed on myelomonocytic cells, binds both classical and nonclassical class I molecules with broad specificity, recruits SHP-1 protein tyrosine phosphatase, and mediates a negative signal that inhibits early signaling events. ILT5 also delivers inhibitory signals but apparently does not interact with any of the class I molecules available for testing. Thus, activation of myelomonocytic cells appears to be negatively regulated by a variety of inhibitory receptors with distinct ligands, some of which are still unknown.

Materials and Methods

Cells

NK cells, EBV-transformed B cell lines, monocytes, macrophages, dendritic cells (DCs), MHC class I-deficient 721.221 cells, and HLA-A*0301-, -B*2705, -Cw*0301, -Cw*0501, and -G1 transfectants of 721.221 were obtained and cultured as previously reported (8, 9). Peripheral B cells were cultured and activated in the presence of CD40L-transfected J558L mouse myeloma cells (10).

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[3] Abbreviations used in this paper: Ig-SF, Ig-superfamily; DC, dendritic cells; ILT, Ig-like transcript; ITIM, immunoreceptor tyrosine-based inhibitory motif; RBL, rat basophilic leukemia; PE, phycoerythrin; SAV-PE, streptavidin-PE; TNP, 2,4,6 trinitrophenyl; SHP, SH2-containing protein tyrosine phosphatase; SHIP, SH2-containing inositol phosphatase.

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Transfections

ILT4 and ILT5 cDNAs were subcloned into pFLAG-CMV-1 (Kodak, Rochester, NY) and transfected either transiently in COS7 cells or stably in rat basophilic leukemia (RBL) cells, as described (8). ILT4 and ILT5 cell surface expression was checked by FACS analysis after staining with the anti-FLAG peptide M2 mAb (Kodak).

Production of ILT4, ILT5, and MHC class I soluble proteins

The extracellular regions of ILT4 and ILT5 were produced as soluble proteins containing the *myc* epitope and a polyhistidine metal-binding domain at the carboxyl terminus of the protein, using the pSecTag system (Invitrogen, Carlsbad, CA). Streptavidin-PE (SAV-PE)-conjugated HLA-A2, -B8, -B27, and -B35 tetramers were produced as previously described (11).

Binding assays

HLA class I-transfected and -untransfected 721.221 cells were incubated with soluble ILT4 or ILT5, and binding was detected by FACS analysis, using the anti-*myc* epitope 9E10 mAb and/or an anti-oligohistidine mAb (Invitrogen), followed by PE-labeled goat anti-mouse IgG Ab (Southern Biotechnology, Birmingham, AL). ILT4- and ILT5-transfected COS7 cells and untransfected cells were incubated with PE-conjugated HLA-A2, -B8, -B27, and -B35 tetramers for 1 h at 4°C and analyzed by FACS.

Serotonin release

ILT4- and ILT5-transfected and untransfected RBL cells were pulsed with [^{3}H]hydroxytryptamine (NEN, Boston, MA) and plated in 96-well flat-bottom plates precoated with 20 μg/ml of mouse anti-TNP IgE (PharMingen) alone or in combination with either 20 μg/ml of the anti-FLAG M2 mAb or 20 μg/ml of control IgG1 mAb (mouse anti-human CD38, PharMingen, San Diego, CA). In another set of experiments, [^{3}H]hydroxytryptamine-pulsed cells were plated in 96-well flat-bottom plates containing either HLA-class I-transfected or untransfected 721.221 cells coated with TNP and mouse anti-TNP IgE. Serotonin release was measured as described (8). To coat cells with TNP-IgE, cells were first incubated with glutamyl-glutamic-ether-phosphatidylethanolamine (Glu2-EPE)-conjugated TNP (kindly provided by Dr. Weltzien, Max Planck Institute, Freiburg, Germany) (12) and then with mouse anti-TNP IgE (20 μg/ml).

Immunoprecipitations and immunoblottings

ILT4 was immunoprecipitated from ^{125}I surface-labeled ILT4-transfected RBL cells with anti-FLAG M2 mAb and analyzed by standard SDS-PAGE under reducing conditions. In immunoblotting experiments, ILT4 was immunoprecipitated with anti-FLAG M2 mAb from ILT4-transfected RBL cells either stimulated with pervanadate or unstimulated as described (8). Precipitates were resolved by SDS-PAGE under nonreducing conditions, transferred to nitrocellulose membranes, and immunoblotted with anti-SHP-1, anti-SHP-2 (Santa Cruz Biotechnology), or with anti-SHIP (kindly provided by Dr. Mark Coggeshall, Ohio State University, Columbus, OH) rabbit antisera as previously described (8).

RT-PCR and oligonucleotide primers

An ~800-bp fragment of ILT4 fragment was amplified from different RNAs by RT-PCR, as previously described (6). Amplification primers were as follows: sense, ACCCCCTGGACATCCTGATCAC; antisense, TGGAGTCTGCGTACCCTCC. Amplified ILT4 cDNA fragments were separated by electrophoresis, transferred to a nylon membrane, and hybridized with the ILT4-specific ^{32}P-labeled oligonucleotide GGATGTTG GAAATCAGCCTT. In control experiments, a ~300-bp cDNA fragment of β-actin was amplified by RT-PCR from the same RNAs (6).

Production of anti-ILT4 mAbs and FACS analysis

Ten-wk-old Wistar rats were immunized three times with ILT4-transfected RBL cells. 42D1 mAb was selected by flow cytometry for staining ILT4-transfected RBL cells, as compared with ILT5-transfected and untransfected RBL cells.

Measurement of cytosolic calcium

Cells were loaded with Indo-1 AM (Sigma, St. Louis, MO) and then incubated for 5 min on ice with the anti-ILT4 mAb 42D1. The IV.3 mAb specific for FcγRII (HB217, American Type Culture Collection, Rockville, MD) or the 3.8B1 mAb (mouse anti-human HLA-DR (9)) were added, and incubation was continued for 15 min on ice. After washing with RPMI 1640-5%FCS, cells were shifted to 37°C for 15 min followed by addition of 10 μg of a cross-linking Ab (goat anti-mouse IgG+IgM (H+L, cross-reactive with rat Ig; Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were analyzed on a flow cytofluorometer as described (9).

Results and Discussion

ILT4 binds MHC class I molecules

To test whether ILT4 interacts with HLA-class I molecules, we analyzed binding of a soluble ILT4 protein to class I transfectants in 721.221 cells by flow cytometry. As shown in Figure 1*A*, ILT4 bound to HLA-A and -B molecules, and to the non classical class I molecule HLA-G1; no binding was detected with HLA-Cw3, -Cw5-transfected or untransfected cells. To further demonstrate interaction between ILT4 and class I molecules, we tested whether soluble class I molecules bind to ILT4-transfected cells. Soluble HLA-A*0201, -B*0801, -B*2705, and -B*3501 tetramers complexed either with influenza- or HIV-derived viral peptides (11) bound ILT4-transfected COS cells (Fig. 1*B*) but not untransfected COS cells (data not shown). We also tested whether a soluble ILT5 protein binds to class I transfectants (data not shown) and whether class I tetramers bind ILT5-transfected COS cells (Fig. 1*C*). However, no significant binding of ILT5 with class I molecules was detected in any of these assays. These data demonstrate that ILT4 (but not ILT5) binds HLA-class I molecules, apparently with a broad specificity for both classical and nonclassical class I molecules.

ILT4 expressed in RBL cells delivers a negative signal that inhibits serotonin release

To determine whether ILT4 can inhibit cell activation triggered via a stimulatory receptor, ILT4 was stably expressed as an amino-terminal FLAG peptide fusion protein in RBL cells, which release serotonin upon engagement of the Fc receptor for IgE (FcϵRI) (8). As shown in Figure 2*A*, secretion of serotonin triggered via the FcϵRI was inhibited when ILT4 was coengaged, using the anti-FLAG peptide Ab immobilized on plastic to mimic the ILT4 ligand. Similar results were obtained using ILT5-transfected RBL cells, indicating that ILT5 delivers an inhibitory signal as well. To demonstrate that ILT4 delivers a negative signal when engaged with its physiologic class I ligands, ILT4-transfected RBL cells were incubated with either class I-transfected or untransfected 721.221 cells coated with TNP and mouse anti-TNP IgE. As shown in Figure 2*B*, serotonin secretion was reduced when ILT4-RBL cells were incubated with TNP-IgE-coated cells expressing either HLA-A*0301 or HLA-B*2705, but not HLA-Cw*0301. In contrast, no inhibition of serotonin release was observed by incubating ILT5-transfected-RBL cells with TNP-IgE-coated class I transfectants, confirming that ILT5 does not interact with class I molecules, at least those used in these assays.

ILT4 is an ~95-kDa molecule associated with SHP-1 phosphatase

Since the cytoplasmic tail of ILT4 contains 3 tyrosine-based motifs similar to those that recruit SHP-1 phosphatase in ILT2 (7, 8), we determined whether ILT4 also recruits phosphatases. ILT4 was immunoprecipitated from ^{125}I-labeled ILT4-transfected RBL cells using the M2 mAb as a broad band of ~95 kDa under reducing conditions (Fig. 3, upper panel). The heterogeneity of ILT4 was most likely due to different degrees of glycosylation. ILT4 was then immunoprecipitated from unlabeled ILT4-transfected RBL cells either unstimulated or stimulated with pervanadate. Immunoprecipitates were immunoblotted with anti-SHP-1, anti-SHP-2, and anti-SHIP Abs. As shown in Figure 3 (lower panel), ILT4 was constitutively associated with SHP-1, and this association was increased after treatment with pervanadate, whereas no association was detected with SHP-2 or SHIP (data not shown).

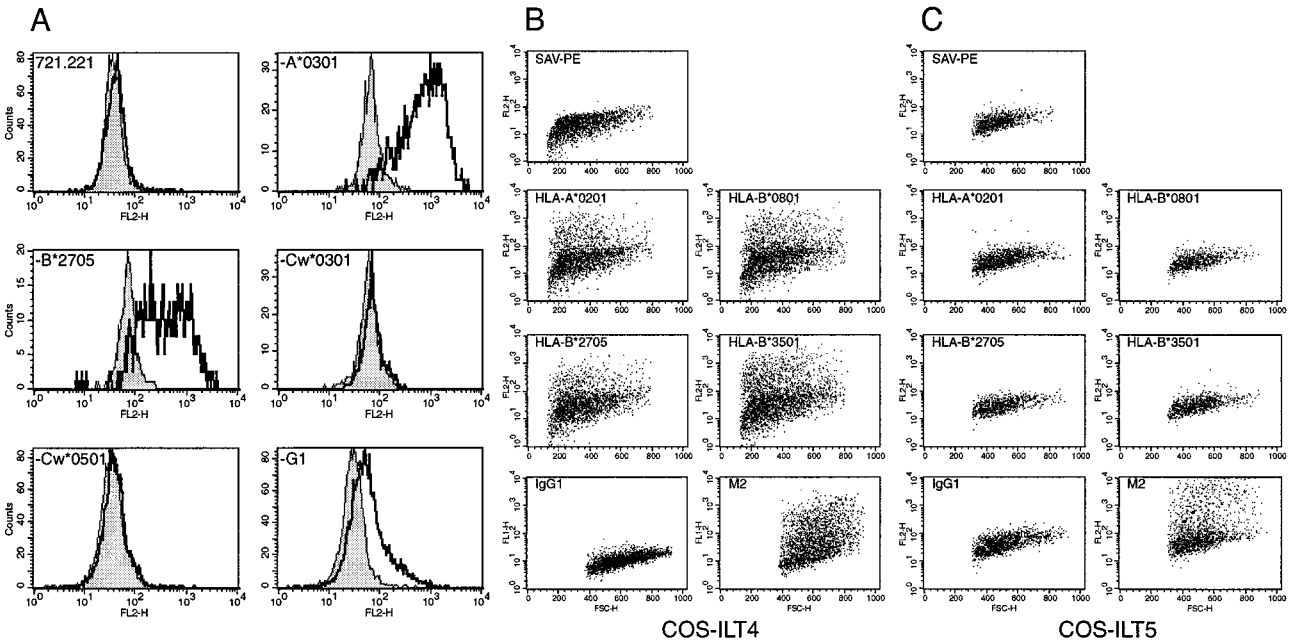

FIGURE 1. *A*, ILT4 soluble protein binds HLA-A*0301, -B*2705, and -G1 transfectants, but not HLA-Cw*0301, -Cw*0501 and untransfected 721.221 cells. Cells were incubated either with (bold profiles) or without (shaded profiles) a soluble ILT4 fusion protein, followed by an anti-*myc* epitope or an anti-oligohistidine mAb and by PE-labeled goat anti-mouse IgG Ab. Binding was analyzed by FACS analysis. The level of class I expression on transfectants was determined by FACS analysis with W6/32 mAb and did not correlate with binding of ILT4. A soluble ILT5 fusion protein did not bind to any of the class I transfectants (data not shown). *B*, HLA-class I soluble proteins bind ILT4-transfected COS cells. ILT4 transiently transfected COS cells were incubated with soluble HLA-A*0201, -B*0801, -B*2705, and -B*3501 labeled with SAV-PE. Binding was assessed by FACS analysis. SAV-PE clone was used as negative control. ILT4 expression on transfected COS cells was determined in the same experiment by FACS analysis using the anti-FLAG peptide mAb (M2). *C*, HLA-class I tetramers did not bind to ILT5-transfected COS cells.

ILT4 is selectively expressed in myelomonocytic cells and inhibits early activation events

The precise pattern of expression of ILT4 was determined by RT-PCR. An ILT4 cDNA fragment was clearly detected in PBMC, monocytes, macrophages derived from purified monocytes, immature DCs, and DCs stimulated either with bacterial products, inflammatory cytokines, or via CD40-CD40L interactions to induce maturation (Fig. 4, *top panel*). Strong expression was also detected in the bone marrow cells. No expression was found in NK cells, T cells, EBV-transformed B cell lines (Fig. 4, *top panel*), peripheral

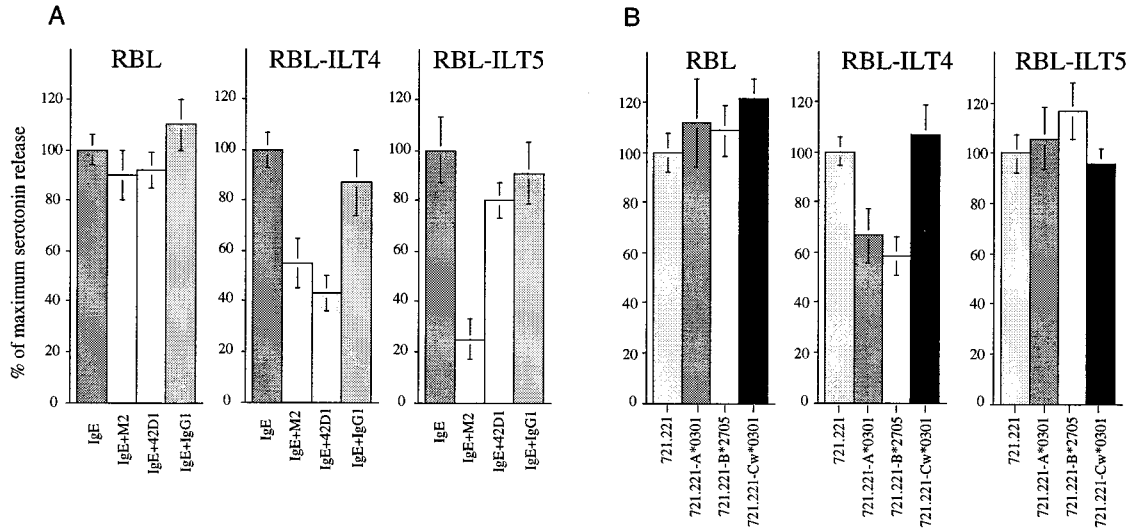

FIGURE 2. Engagement of ILT4 by Ab or class I ligands inhibits IgE-induced serotonin release in ILT4-transfected RBL cells. *A*, ILT4-, ILT5-transfected and control RBL cells were stimulated with purified mouse IgE (20 μg/ml) alone and in combination either with the M2 mAb (20 μg/ml) or with the anti-ILT4 42D1 mAb (20 μg/ml) or with an IgG1 control Ab (20 μg/ml) immobilized on plastic (for description of 42D1 mAb see Fig. 4 and 5). *B*, ILT4-, ILT5-transfected and untransfected RBL cells were conjugated with either HLA-A*0301, or HLA-B*2705-, or HLA-Cw*0301-transfected or untransfected 721.221 cells, coated with TNP and mouse anti-TNP IgE. Serotonin release is expressed as percentage of maximum serotonin release obtained with mouse anti TNP-IgE (*A*) or TNP-IgE coated 721.221 cells (*B*).

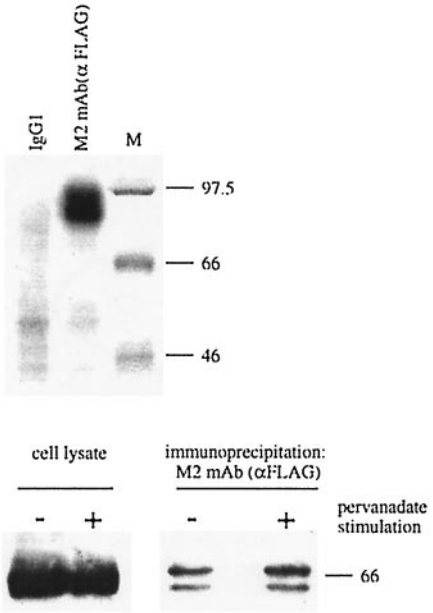

FIGURE 3. ILT4 is a ~95-kDa glycoprotein associated with SHP-1. *Top panel*, ILT4 tagged with an N-terminal FLAG peptide is immunoprecipitated from ^{125}I-labeled ILT4-transfected RBL cells with the M2 mAb as a 95-kDa band in SDS-PAGE under reducing conditions. No bands were detected using a control IgG1 mAb as immunoprecipitating Ab. *Bottom panel*, ILT4 is associated with SHP-1. ILT4-transfected RBL cells were incubated for 10 min at 37°C either with medium alone or with pervanadate. ILT4 was then immunoprecipitated with the M2 mAb, and immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-SHP-1 Ab. Whole cell lysates were included as a positive control. SHP-1 association with ILT4 is increased after cell stimulation with pervanadate.

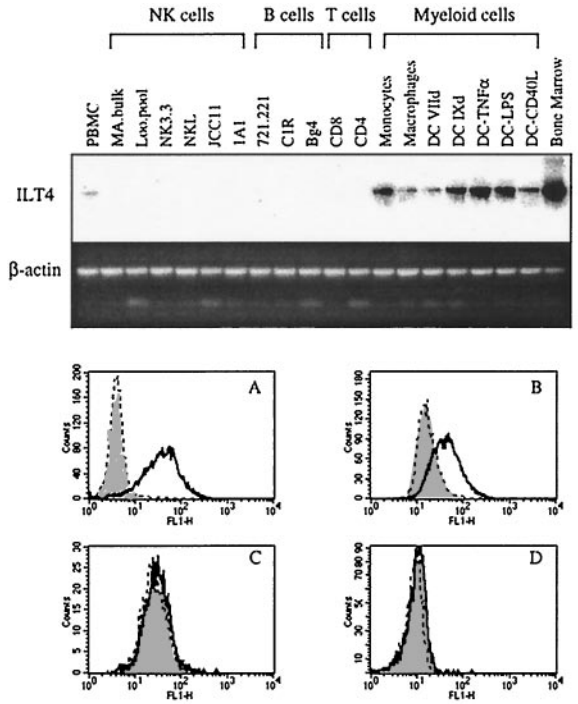

FIGURE 4. ILT4 cDNA is expressed in myelomonocytic cells. *Top panel*, An ILT4 cDNA fragment is detectable by RT-PCR in PBMCs, monocytes, macrophages, immature (VIId and XId), and mature DCs (TNF-α, LPS, CD40L), and bone marrow cells, whereas no bands are detectable in NK cells, B cell lines, and T cells. *Bottom panel*, 42D1 mAb, which is specific for ILT4, stains peripheral blood monocytes (*A*) and DCs (*B*), whereas no reactivity is observed on peripheral activated B cells (*C*) (10), and NK polyclonal cells (*D*). Shaded profiles illustrate staining with an isotype-matched control Ab.

B cells, or neutrophils (data not shown). To determine whether ILT4 is capable of an inhibitory function in myelomonocytic cells, we produced an anti-ILT4 mAb, using ILT4-transfected RBL cells as immunogen. Anti-ILT4 42D1 mAb stained monocytes and DCs, but not purified peripheral B cells, NK cells (Fig. 4, *bottom panel*), and T cells (data not shown), confirming the expression pattern previously demonstrated by RT-PCR. Using the 42D1 mAb as ligand for ILT4, we tested whether coengagement of ILT4 can inhibit Ca^{2+} mobilization triggered via the FcγRII (13) and HLA-DR (9). Co-cross-linking of ILT4 with FcγRII (Fig. 5) or HLA-DR (data not shown) induced a clear reduction of the increased $[Ca^{2+}]_i$ triggered through these receptors in DCs. These results demonstrate that engagement of ILT4 can negatively modulate signaling in myelomonocytic cells.

Conclusions

Our results show that ILT4 is selectively expressed in myelomonocytic cells, binds MHC class I molecules, and inhibits early signaling events triggered via stimulatory receptors. At present, it is not known which cellular functions are controlled by ILT4. ILT4 may modulate one or several of the antigen presenting functions mediated by myelomonocytic cells, such as antigen uptake and presentation, migratory capacity, cytokine production, and costimulatory function (14). ILT4 may also control inflammatory responses mediated by monocyte-macrophages, such as oxidative burst, or inhibit their cytotoxicity, enabling them to recognize tumor cells that have lost expression of self-class I molecules (15, 16). It is noteworthy that ILT4 binds HLA-G, a nonclassical class I molecule selectively expressed in the trophoblast, a tissue of fetal origin devoid of HLA-A and -B molecules that separates the developing embryo from the mother. The recognition of HLA-G by ILT4 may play a role in maternal tolerance against the fetal semiallograft.

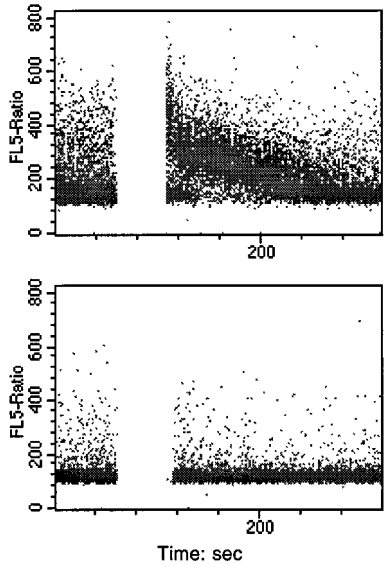

FIGURE 5. Intracellular Ca^{2+} mobilization is induced in DCs by the IV.3 mAb specific for the stimulatory isoform of the FcγRII (*upper panel*). Cross-linking of FcγRII with ILT4 abolishes Ca^{2+} mobilization (*lower panel*).

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