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Proper Regrafting of Ig-Like Transcript 2 after Trogocytosis Allows a Functional Cell–Cell Transfer of Sensitivity

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The acquisition by T cells of exogenous ligands originally expressed by APC has been already described. However, reports essentially focused on the outward signaling of acquired ligands and their effects on surroundings cells. We investigated the function of transferred receptors (not ligands) on the T cells that acquired them (not on cells they interact with). We show that inhibitory Ig-like transcript 2 receptors efficiently transfer from monocytes to autologous T cells by trogocytosis and integrate within the plasma membrane of the acquirer T cells. Furthermore, the acquired receptors can access compatible signaling machinery within acquirer T cells and use it to signal and alter the functions of their new host cells. These data are a formal demonstration that a transferred molecule may send signals to its new host cell. We also provide evidence that sensitivity to modulatory molecules can be acquired from other cells and introduce the notion of intercellular transfer of sensitivities. The Journal of Immunology, 2011, 186: 2210–2218.

Trogocytosis is a mechanism of fast cell-to-cell contact-dependent uptake of membranes and associated molecules from one cell by another (1). Intercellular transfers of membrane patches are observed during interactions between immune cells (reviewed in Ref. 2). Trogocytosis of ligands is well established, in vitro in humans and also in vivo in mice (3–8), in which MHC class I, MHC class II, and costimulatory molecules can transfer from APC to T cells. The ligands acquired through trogocytosis may temporarily transfer functions of the donor cells to the acceptor cells. For example, 1) CD8+ T cells that acquired their MHC class I ligands became the targets of fratricide Ag-specific cytolysis (3); 2) T cells that acquired HLA-DR and CD80 could stimulate resting T cells (2); and 3) CD4+ T cells that acquired HLA-G behaved as suppressor cells (9). Hence, acquisition of exogenous ligands by T cells endows them with new outward signaling properties and enables them to influence the functions of surrounding immune cells.

Transfers of receptors and inward signaling through acquired receptors have been much less studied, even though work has started in this direction. For instance, it has been shown that constitutively activated molecules that were acquired by intercellular transfers may signal to their acquirer cells. However, in these reports involving cell lines, receptor–ligand interactions played no role because of the constitutive activation of the transferred molecules (10, 11). By contrast, a recent report investigated the functional consequences of the trogocytic transfer of FcRs from murine APC to murine T cells. In this report, it was shown that FcRs could bind with their ligands after transfer, but could not transduce signals to their new host cells (12). Hence, the capability of a transferred receptor to bind to its ligand, transduce a signal, and function remains to be demonstrated, especially for nontransformed human cells. This requires the demonstration of: 1) the proper insertion of the acquired receptor within the plasma membrane of the new host cell; 2) the capability of the transferred receptor to access the intracellular machinery of the new host cell; and 3) the functional consequences of its binding to a ligand.

In this study, we investigated these issues using the transfer of the Ig-like transcript 2 (ILT2) inhibitory receptor (LILRB1/CD85j) from monocytes to ILT2-negative autologous T cells. The T cells we used were ILT2-negative, but nevertheless, it has been shown that in cases in which ILT2 is expressed by T cells, ILT2 engagement inhibits their proliferation and cytolytic functions (13). In these cases, ILT2 inhibitory functions are mediated by tyrosine phosphorylation of ITIM-like sequences in its cytoplasmic tail and involve the p56lck kinase and recruitment of Src homology region 2 domain-containing phosphatase-1 and -2 phosphatases (14). In ILT2-transfected cells, ILT2 phosphorylation could be induced by cross-linking or by the protein tyrosine phosphatase inhibitor pervanadate (PV). ILT2 signaling depends on binding to HLA class I molecules, and ILT2’s highest affinity ligand is HLA-G (for a review on HLA-G, see Ref. 15).

In this study, we demonstrate the ILT2 transfers from monocytes to autologous activated T cells by trogocytosis and the proper integration of transferred ILT2 within the plasma membrane of a new host cell. Furthermore, we show that transferred ILT2 behaved as an endogenously produced molecule, capable of using the intracellular biochemical machinery of its new cell and send inward-oriented signals, which are then responsible for the acquirer T cell functional inhibition. These data are a formal demonstration that a transferred molecule may send signals to its new host cell after ligand engagement. They imply that sensitivity to modulatory molecules can be acquired from other cells.

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Abbreviations used in this article: GAM, goat anti-mouse; ILT2, Ig-like transcript 2; LCL, lymphoblastoid cell line; PV, pervanadate.

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Materials and Methods

Cells and cell lines

Blood was obtained from healthy volunteers from French Blood Bank under a protocol approved by the Institutional Review Board of the Saint-Louis Hospital, Paris, France.

For all purification steps, FcRs on PBMC were blocked using 20 μg/ml human IgG (Sigma-Aldrich) for 30 min.

For monocYTE isolation, PBMCs were labeled with 20 μg/ml anti-CD14 and then positively separated using goat anti-mouse (GAM)-coated magnetic beads according to the manufacturer’s specifications (Ademtech). To obtain highly purified CD4 + or CD8 + T cells, PBMCs were labeled with anti-CD4 or anti-CD8 and then positively separated using GAM-coated magnetic beads. Positively isolated cells were then incubated overnight at 37°C and repeatedly washed to increase the purity. To obtain negatively purified CD4 + or CD8 + T cells, PBMCs were labeled with a mixture of anti-CD4, anti-CD14, anti-CD19, and anti-CD8 or anti-CD4 followed by immunomagnetic depletion using GAM-coated magnetic beads. ILT2-positive cells were removed using 20 μg/ml purified blocking Ab against ILT2 (GHI/75) and then positively separated using GAM-coated magnetic beads. ILT2 expression was evaluated by flow cytometry.

Cell lines used were previously described (9).

Cell activation

Purified monocytes were activated with 100 ng/ml LPS (Sigma-Aldrich) for 3–5 d. PBMCs or purified T cells were activated for 48 h with 4 μg/ml leucoagglutinin (PHA-L; Sigma-Aldrich) and then cultured in IL-2-supplemented medium (100 U/ml; Sigma-Aldrich) for 2–4 more d prior to use. Cells were not used beyond these times.

Abs and flow cytometry experiments

The following Abs were used from Exbio (Prague, Czech Republic): purified anti-CD4, -CD8, -CD14, and -CD19, blocking anti-HLA-G 87G Fab, non-blocking anti-HLA-G Mem-G9, and FITC-conjugated anti-CD4, -CD8, and -CD14; from Beckman Coulter: purified anti-CD28, anti-CD16, PC5-conjugated anti-CD3, and anti-CD25, from BD Biosciences: purified and PE-conjugated anti-ILT2 (clone GHI/75); from Santa Cruz Biotechnology: purified anti-ILT2 (clone VMP55); from Invitrogen: Alexa Fluor 488 GAM IgG1; and from Upstate Biotechnology: anti-phosphotyrosine 4G10. Purified anti-CD3 (OKT3) was provided by Janssen-Cilag.

For flow cytometry experiments, FcRs were blocked in 25% human serum supplemented with 20 μg/ml human IgG serum prior to staining. All FcR blocking conditions were maintained during the entire duration of the experiments. Appropriate isotypic controls were systematically used to evaluate nonspecific binding. PKH67 or PKH26 dyes (Sigma-Aldrich) were used for fluorescent labeling of cell membranes and the sNHS-Biotin kit (Upima) was used for cell-surface biotinylation according to the manufacturers’ specifications.

Confocal microscopy

T cells were conjugated with PKH26-labeled monocytes at 37°C and left adhered on poly-l-lysine-coated slides for 5 min at 37°C. The cells were then fixed for 10 min with 3% paraformaldehyde, stained using an anti-ILT2 (VMP55) followed by GAM Alexa Fluor 488, and analyzed using a Carl Zeiss LSM 510 confocal microscope (Carl Zeiss).

Trogocytosis assays

Trogocytosis assays were performed as previously described (9). Briefly, highly purified CD4 + and CD8 + T cells were cocultured for 30 min with purified autologous monocytes at a 1:1 ratio, to a total concentration of 10 6 to 10 8 cells/ml, at 37°C in a 5% CO 2 humidified incubator. All further steps were performed on ice.

For acid wash, cells were washed twice in PBS and resuspended for 4 min at 20°C in citrate buffer (0.133 M citric acid and 0.066 M Na 2HPO 4, pH 3.3) at a density of 5 × 10 6 cells/ml. The treatment was stopped by addition of 10% FCS in RPMI 1640 and 10 mM HEPES.

Surface biotinylation experiments

Prior to the coincubation with autologous T cells, the monocytes’ cell-surface proteins were biotinylated using sNHS-Biotin (Upima). At the end of the coincubation, T cells were purified, lysed, and the presence of biotinylated ILT2 was investigated by immunoprecipitation of biotinylated proteins with streptavidin-agarose beads (Sigma-Aldrich) followed by electrophoretic separation on a 10% SDS-PAGE acrylamide gel, transfer to nitrocellulose membranes, and immunoblotting with anti-ILT2 (VMP55 clone).

ILT2 phosphorylation analysis

Cells treated with the phosphatase inhibitor PV (200 μM sodium orthovanadate and 200 μM H 2 O 2 at 37°C for 10 min) were lysed in 1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 0.5% Nonidet P-40, protease inhibitors (Sigma-Aldrich), and phosphatase inhibitors cocktails I and II (Sigma-Aldrich). Lysates were precleared with protein G-Sepharose beads (Amersham Biosciences) and subjected to immunoprecipitation with 2 μg purified anti-ILT2 (clone GHI-75; BD Biosciences) conjugated to protein G-Sepharose beads. Immunoprecipitates were separated by standard SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Immunoblotting was performed with anti-phosphotyrosine (Upstate Biotechnology) or anti-ILT2 followed by GAM-HRP (Sigma-Aldrich) or with streptavidin-HRP (Sigma-Aldrich). The membrane was developed using ECL + Western blotting detection reagents (Amersham Biosciences).

HLA-G–ILT2 blocking procedures

For blocking HLA-G–ILT2 interactions, FcRs were first blocked by incubation of the cells 30 min in 25% human serum supplemented with 20 μg/ml human IgG. Cells were then incubated with 10 μg/ml blocking Ab against HLA-G 87G or nonblocking anti-HLA-G M8 (Amersham Pharmacia). Ab anti-ILT2 GHI/75, or their isotypic controls, prior to use. All blocking conditions were maintained during the subsequent experiments.

Proliferation assays

Proliferation was measured by tritiated thymidine ([ 3 H]thymidine) incorporation (1 μCi/well; Amersham Biosciences) on a β-counter (Wallac 1450; Amersham Biosciences). All samples were run in triplicate. Appropriate proliferation and isotypic controls were included for each experiment on each plate.

Measure of the ongoing proliferation of already activated cells. A total of 5 × 10 6 proliferating cells (CD4 +ILT2Acq + or CD4 + T cells) was cocultured with 2.5 × 10 6 cells gamma-irradiated HLA-G–expressing cells (lymphoblastoid cell line (LCL)–HLA-G1 (75 Gy) and their negative counterparts LCL-RSV and plated in a final volume of 200 μl. [ 3 H]thymidine was added at the time of incubation and proliferation was measured 18 h later.

Alloproliferative responses of resting cells. A total of 5 × 10 4 responder naïve PBMCs or highly purified CD4 + T cells from these PBMCs or highly purified CD4 + T cells incubated with autologous monocytes at a 1:1 ratio (CD4 + T cells plus monocytes) were stimulated with 2.5 × 10 5 gamma-irradiated LCL–RSV cells or LCL–HLA-G1 cells (75 Gy). Proliferation was measured from day 1–6 every 24 h. Separate plates were set up for each time point, and [ 3 H]thymidine was added 18 h before collection.

Cytotoxicity assays

Cytolytic activity of CD8 + T cells was assessed in a standard 4 h [ 51 Cr] release assay against 51Cr-labeled HLA-G-negative melanoma M6 cells transfected by a control vector (M6-pcDNA) or the same vector containing HLA-G1 cDNA (M6–HLA-G1). (M6 cells and the inhibition of CTL killing are detailed in Refs. 16, 17). Briefly, effector cells were mixed with 5 × 10 5 51Cr-labeled target cells (Amersham) at different E:T ratios in 80–100 μl microtitrator plates. After 4 h of coincubation at 37°C in a humidified 5% CO 2 incubator, supernatant was collected for liquid scintillation counting (Wallac 1450 Microbeta; Pharmacia). Percentage of specific lysis was determined as (cpm experimental well – cpm spontaneous release)/ (cpm maximum release – cpm spontaneous release) × 100. Spontaneous release was determined by incubation of labeled target cells with medium. Maximum release was determined by solubilizing target cells in 1% Triton X-100. For each experiment, triplicate samples were used.

Statistical analysis

Data are presented as means ± SD. Student t test was used, and a p value <0.05 was taken to be significant. For figures showing representative experiments, error bars represent SD of triplicates.

Results

T cells require ILT2-positive cells to upregulate cell-surface ILT2 upon activation

ILT2 can be expressed at the surface of a minor fraction (0–25%) of resting CD4 + and CD8 + T cells in healthy individuals (13, 18), and this proportion is increased in activated and Ag-specific T cells.
ILT2 on CD4+ and CD8+ T cells is acquired from monocytes by membrane transfers (trogocytosis)

Out of all the possible explanations of the fact that monocytes are required for activation-dependent T cell surface expression of ILT2, we show in this study that ILT2 is actually not upregulated by T cells themselves, but acquired from monocytes by T cells through the transfer of ILT2-containing membrane patches, a mechanism known as trogocytosis.

The hallmarks of trogocytosis are kinetics in the order of minutes, transfer of membrane patches and not of individual molecules, cell-cell contact dependence, and a limited lifespan of the acquired molecules at the surface of the acquirer cell (1, 2). Fig. 2A shows that when monocytes for which membranes had been labeled with a lipophilic dye (PKH67) were coincubated with activated autologous CD4+ T cells for 30 min or less, a correlation between T cell positivity for PKH67 and ILT2 was observed. This shows that ILT2-positive T cells had acquired membranes from monocytes. Supplemental Fig. 2A shows that ILT2 acquisition was dependent on cell-to-cell contact, and Supplemental Fig. 2B shows the limited lifespan of acquired ILT2 at the T cell surface, which indicates that ILT2 was not endogenously produced by T cells. Real-time quantitative PCR experiments also indicated that ILT2-negative activated CD4+ T cells expressed very low amounts of ILT2 mRNA prior to use and that these levels were not significantly increased after trogocytosis experiments (data not shown). Moreover, in accordance with previously published data (21), Supplemental Fig. 3 shows that CD4+ and CD8+ T cells acquire ~1% of membranes of monocytic origin and between 1 and 0.5% of monocytic ILT2 proteins after a 30-min coincubation with autologous monocytes. Fig. 2B illustrates by confocal microscopy that the only ILT2 expressed by CD4+ T cells after coincubation with autologous monocytes, Fig. 2B demonstrates that only ILT2 detectable on T cells was transferred ILT2. These data demonstrate that T cells can acquire ILT2 receptors from autologous monocytes by trogocytosis.

Proper insertion of membrane ILT2

Membranes transferred from a cell to another may not integrate, but may remain affixed onto the acceptor cell (i.e., outside). Obviously such a transfer is unlikely to allow signaling of the acquired molecules to the acquirer cell (2, 22). Membranes that are affixed on acceptor cells can be removed by a mild acid wash treatment (pH 3.3) (23). A pH 3.3 treatment (Fig. 3A) efficiently removed β2-microglobulin, leading to a loss of staining for HLA class I Ags using an Ab directed against conformed HLA class I molecules (W6/32). However, this treatment did not remove the PKH-labeled membranes of monocytic origin, or the ILT2 they contained, from the CD4+ T cell surface. This ruled out the possibility that the acquired membranes were just affixed onto the surface of T cells.

If properly inserted, transferred membranes and molecules should spatially behave as endogenously produced ones, and move, or diffuse within the acceptor cell’s plasma membrane. Fig. 3B shows by confocal microscopy a representative example of our observations. Early within the first 30 min of coincubation, most of the transferred ILT2 at the surface of CD4+ T cells remained within patches of PKH-labeled monocytic membranes. At later times, ILT2 receptors were also observed mainly within PKH-labeled monocytic membrane patches. However, an increasing proportion of cells that showed ILT2 receptors outside PKH-labeled membranes of monocytic origin was observed when incubation time increased. Representative images are shown in Fig. 3B. Three-dimensional images of T cells with PKH-free ILT2 are provided in Supplemental Video 1.
The independent movements of dyed lipids and ILT2 show that at least some ILT2 + monocytic membranes integrated properly, even though we cannot know whether ILT2 molecules exited the original membrane patch, or whether labeled lipids diffused out of the area beneath ILT2.

Transferred ILT2 can access the intracellular machinery of their new cell host

It was shown that ILT2 phosphorylation is induced in T cells by the protein tyrosine phosphatase inhibitor PV (14). Thus, we coinubated activated CD4+ T cells and biotinylated monocytes, CD4+ FIGURE 2. ILT2 display by activated T lymphocytes is due to trogocytosis. A, Activated CD4+ T cells that display ILT2 also display membranes acquired from autologous monocytes. Flow cytometry was performed on purified activated CD4+ T cells prior to or after a 30-min coinubation with autologous monocytes, the membranes of which had been labeled with the lipophilic dye PKH67. Data show the presence of membranes of monocytic origin versus ILT2 on CD4+ T cells. Percentage of ILT2+PKH67+ double-positive CD4+ T cells is indicated. Experiment shown is representative of three. B, ILT2 and membranes acquired from monocytes are colocalized on activated CD4+ T cells. Confocal microscopy was performed on purified activated CD4+ T cells after a 30-min coinubation with autologous monocytes, the membranes of which had been labeled with the lipophilic dye PKH26. Blue, nuclear staining (DAPI); green, ILT2; red, monocyte membranes. Scale bars, 5 μm. Data show the colocalization of monocytic membranes and ILT2, indicative of a monocytic origin of ILT2. Left panel, Monocyte–lymphocyte conjugate. Right panel, CD4+ T lymphocyte after coinubation. Arrows point to areas of interest. Experiment shown is representative of three. C, Biotinylated ILT2 is acquired by autologous activated CD4+ T cells. Upper panel, Total ILT2 content in lysates of purified CD4+ T cells and autologous monocytes at the time of coinubation. Immunoprecipitation with anti-ILT2 (clone GHI/75), blotting with anti-ILT2 (VMP55 clone). Lower panel, Detection of monocytic ILT2 in T cell lysates after coinubation with biotinylated monocytes. Immunoprecipitation with streptavidin beads, blotting with anti-ILT2 (VMP55 clone). Biotinylated Monocytes, control biotinylated monocytes prior to coinubation with CD4+ T cells; CD4+-ILT2BiotinAcq+, CD4+ T cells after a 30-min coinubation with biotinylated monocytes; CD4+ T cells, CD4+ T cells prior to coinubation with biotinylated monocytes.

FIGURE 3. ILT2-containing membranes of monocytic origin integrate into CD4+ T cell plasma membrane. A, ILT2-containing membranes of monocytic origin on activated CD4+ T cells are not removed by acid wash. Flow cytometry analysis for ILT2 and PKH67 was performed on CD4+ T cells that had acquired ILT2-containing PKH67-labeled membranes from autologous monocytes. ILT2 cell-surface expression and PKH67 positivity was evaluated prior to and after acid wash. Percentage of ILT2+PKH67+ double-positive CD4+ T cells is indicated. The experiment shown is representative of three. B, ILT2 of monocytic origin can rapidly be observed as PKH-free clusters at the surface of CD4+ T cells. Confocal microscopy for ILT2 and PKH26 was performed on purified activated CD4+ T cells that had acquired ILT2-containing PKH26-labeled membranes from autologous monocytes. Scale bars, 5 μm. Blue, nuclear staining (DAPI); red, membranes of monocytic origin (PKH26); green, ILT2 (of monocytic origin). Left panel, CD4+ T lymphocyte prior to trogocytosis. Middle panel, CD4+ T lymphocyte during a coinubation with autologous monocytes. Right panel, Examples of PKH26 versus ILT2 localization on CD4+ T cells. The arrows point to areas of interest.
T cells that had acquired ILT2 receptors (CD4\(^+\)ILT2\(^{\text{BiotinAcq+}}\)) were then sorted, treated with PV, lysed, and the phosphorylation of acquired ILT2 receptors was determined by Western blotting with streptavidin-HRP or antiphosphotyrosine. Fig. 4 shows once again that CD4\(^+\) T cells did not express endogenous ILT2 prior to coincubation with monocytes and acquired biotinylated ILT2 during the coincubation with biotinylated autologous monocytes. Fig. 4 also shows that PV treatment had no effect on the level of acquired ILT2 in T cells, but was able to induce ILT2 phosphorylation in CD4\(^+\)ILT2\(^{\text{BiotinAcq+}}\) T cells. This demonstrates that acquired ILT2 receptors were accessible to the biochemical machinery of the acceptor T cells. Purity of the T cell population and contamination controls ensured that the observed ILT2 phosphorylation was not related to the presence of contaminating monocytes.

Thus, these data demonstrate that acquired ILT2 receptors are properly inserted within the plasma membrane of T cells, can diffuse within the plasma membrane, and that transferred ILT2 was accessible to components of the T cell intracellular machinery.

Acquired ILT2 inhibits the function of acceptor T cells after ligand engagement

It was shown that engagement of T cell ILT2 with HLA-G induces inhibition of CD4\(^+\) T cell proliferation (9, 24) and CD8\(^+\) T cell cytolytic function (25). We investigated whether engagement of acquired ILT2 with HLA-G was able to cause T cell functional inhibition.

We first showed that transferred ILT2 receptors were able to mediate the inhibition of CD4\(^+\) T cell ongoing proliferation upon engagement with HLA-G. For this, we generated purified, polyclonally activated ILT2-negative CD4\(^+\) T cells and had them acquire or not ILT2 from autologous monocytes (CD4\(^+\), CD4\(^+\)ILT2\(^{\text{BiotinAcq+}}\) T cells, respectively). These T cells were already proliferating at the start of the experiment, and their original lack of ILT2 was assessed by flow cytometry. HLA-G-expressing cells were then added and T cell proliferation measured. Fig. 5a shows that the proliferation of ILT2-negative CD4\(^+\) T cells was not inhibited by HLA-G, but that of CD4\(^+\)ILT2\(^{\text{Acq+}}\) T cells was completely stopped in the presence of HLA-G. The inhibition of CD4\(^+\)ILT2\(^{\text{Acq+}}\) T cells was directly due to the interaction between acquired ILT2 receptors and HLA-G. Indeed, anti–HLA-G (87G) and anti-ILT2 (GHI/75) blocking Abs, but not nonblocking anti–HLA-G Ab (Mem-G/9), restored the ongoing proliferation of CD4\(^+\)ILT2\(^{\text{Acq+}}\) T cells. Furthermore, proliferation of CD4\(^+\)ILT2\(^{\text{Acq+}}\) T cells was unaffected when HLA-G-negative control cells were used in the coincubation.

We next showed that acquired ILT2 receptors could inhibit CD8\(^+\) T cell cytotoxic function. For this, we set up cytotoxicity assays between purified activated CD8\(^+\) T cells that originally did not express ILT2, and had acquired ILT2 or not from autologous monocytes (CD8\(^+\) and CD8\(^+\)ILT2\(^{\text{Acq+}}\) T cells, respectively) and HLA-G–positive targets. Fig. 5b shows that ILT2-negative CD8\(^+\) T cells lysed their targets regardless of HLA-G. CD8\(^+\)ILT2\(^{\text{Acq+}}\) T cells lysed HLA-G–negative targets but their cytotoxic functions were blocked by HLA-G–expressing target cells. This inhibition was directly due to HLA-G–ILT2 interaction, as shown by the use of blocking Abs against HLA-G (87G) or ILT2. Isotypic controls or the nonblocking anti–HLA-G Mem-G/09 did not restore the cytotoxic activity of CD8\(^+\) T cells against HLA-G–positive target cells.

Thus, these data show that activated ILT2-negative CD4\(^+\) and CD8\(^+\) T cells are intrinsically insensitive to ILT2 ligands (e.g., HLA-G) until they can borrow ILT2 from ILT2-expressing cells such as monocytes by trogocytosis of ILT2-containing membranes. Given that resting T cells have little or no trogocytic capability (9), these data also indicate that T cell inhibition through ILT2 engagement should concern only fully activated T cells.

Inhibition of CD4\(^+\) T cell alloroactivity through ILT2 is directed by ILT2 trogocytosis

We next demonstrated that trogocytosis-dependent ILT2-mediated inhibition held true in situations in which resting T cells were involved. For this demonstration, we studied ILT2-mediated inhibition of resting T cell responsiveness to allogeneic stimulation using HLA-G as ILT2 ligand, because it is known that HLA-G inhibits the alloproliferative response of PBMC, as measured by thymidine incorporation at day 6 or 7 after allostimulation (9, 26, 27).

Thus, we set up alloproliferative experiments between irradiated HLA-G–positive or HLA-G–negative LCL as stimulator cells and freshly isolated PBMC or highly purified CD4\(^+\) T cells from these PBMC (CD4\(^+\) T cells), or highly purified CD4\(^+\) T cells from these PBMC mixed with autologous monocytes (CD4\(^+\) T cells plus monocytes), as responder cells. PBMC used in these experiments were chosen for their lack of ILT2 expression on T cells by flow cytometry performed at the time of isolation. In this system, allostimulation was provided by control or HLA-G–transfected LCL cells, and ILT2 was provided by autologous monocytes. To correlate ILT2 acquisition with alloproliferative inhibition, the ILT2 expression and the proliferation of the responder cells were measured every 24 h for 6 d.

For the first 3 d of the experiment, no difference was observed in the phenotype and proliferation of CD4\(^+\) T cells. Thus, in Fig. 6,
Acquisition of ILT2 is responsible for proliferation and cytolytic inhibitions of activated T lymphocytes. A. Acquisition of ILT2 by activated CD4+ T cells inhibits their proliferation in presence of HLA-G molecules. As shown on flow cytometry plots, proliferation assays were performed between activated ILT2-negative CD4+ T cells, which did not acquire ILT2 (top left panel), or activated ILT2-negative CD4+ T cells, which had acquired ILT2 from monocytes (top right panel), with HLA-G1-positive or -negative LCL cells. Proliferation of activated CD4+ T cells was quantified by thymidine incorporation during 18 h of coculture. B. Acquisition of ILT2 by activated CD8+ T cells inhibits their proliferation in presence of HLA-G1 molecules. A proportion of CD8+ T cells displayed cell-surface ILT2 throughout the 6 d of the experiment from day 0, showing in particular no ILT2 expression on CD4+ T cells. For illustration, day 3 data were arbitrarily chosen but do not differ from day 0, showing in particular no ILT2 expression on CD4+ T cells.

As shown by the first two plots in Fig. 6A, CD4+ T cells within a responder population of total PBMC remained negative for cellsurface ILT2 for 4 d. Consistently, as shown by the first two bar graphs, these T cells remained insensitive to inhibition by HLA-G during the first 4 d of allosimulation. As a consequence, the daily levels of proliferation of these PBMCs were identical regardless of whether HLA-G was present in their environment or not. These data on the first days of PBMC allosimulation clearly show that ILT2 does not inhibit the Ag selection and the initial activation steps of resting T cells or even possibly their first proliferation cycles. After day 4, a proportion of CD4+ T cells displayed cell-surface ILT2, and this proportion increased through day 6 (rightmost two plots of Fig. 6A). This means that a proportion of CD4+ T cells became trogocytic as of day 4 and that this proportion increased through day 6. The proportion of ILT2-positive CD4+ T cells was nevertheless much lower than in Figs. 1–5, but this can be explained by the fact that allogeneic activation such as in Fig. 6 concerns only a fraction of resting T cells, whereas polyclonal activation as in Figs. 1–5 concerns all. The ILT2-positive T cells in Fig. 6 would then correspond to effectors generated through Ag-specific activation. This also means that it took 4 d from a resting T cell to become trogocytic upon allosimulation, which matches the kinetics of trogocytic capability induction through allosimulation and polyclonal activation described in this study and elsewhere (9).

By contrast with the results obtained when total PBMCs were allosimulated, highly purified CD4+ responder T cells, which had no access to ILT2 exogenously produced by monocytes, remained cell-surface ILT2 negative throughout the 6 d of the experiment (Fig. 6B, plots) and also remained mostly insensitive to HLA-G-mediated proliferation inhibition (Fig. 6B, bar graphs). However, sensitivity to HLA-G could be restored if the very same highly purified ILT2-negative CD4+ T cells were coincubated with autologous ILT2-expressing monocytes at a 1:1 ratio (Fig. 6C). In this case, the
Acquisition of ILT2 inhibits alloproliferation of CD4 T cells. Mixed lymphocytes reactions were performed between T lymphocytes and stimulatory cells during 6 d. Proliferation was measured at the indicated time points, and ILT2 expression on CD4+ T cells was concomitantly analyzed by flow cytometry. Mixed lymphocyte reactions were set up with no stimulatory cells (none), irradiated LCL stimulator cells (LCL), or irradiated LCL–HLA-G1 stimulator cells in medium alone (LCL–HLA-G1) or supplemented with an isotype-matched irrelevant Ab (LCL–HLA-G1 + Isotypic Control), a nonblocking anti–HLA-G1 Ab [LCL–HLA-G1 + Anti–HLA-G1 (MEM-G/9)], a blocking anti–HLA-G1 Ab as F(ab’)_2 [LCL–HLA-G1 + Anti–HLA-G1 (87G Fab)], or a blocking anti-ILT2 Ab (LCL–HLA-G1 + Anti–ILT2). The responder cell populations used were total resting PBMCs (A), purified CD4+ resting T cells (B), purified CD4+ resting T cells plus autologous purified monocytes (C) at a 1:1 ratio. Data presented are from one out of three independent experiments performed. *Significant statistical analysis.
kinetics of ILT2 display by CD4+ T cells and those of alloproliferation inhibition matched those of control PBMC.

Thus, these data show that membrane transfers are required for the in vitro ILT2-mediated inhibition of T cell alloproliferative responses and that the parameters of trogocytosis dictate which cell is concerned as well as the kinetics of ILT2-mediated inhibition.

Discussion
A minor proportion of T cells have been shown to be able to express ILT2 at the resting stage, a proportion that was shown to increase with activation and even more so in cloned T cells (29). Because endogenous expression of ILT2 by T cells would preclude any investigation on transferred ILT2, we used ILT2-negative resting CD4+ and CD8+ T cells and autologous monocytes purified from resting PBMCs of healthy donors as the only ILT2-expressing cells. The absence of ILT2 cell-surface and intracellular expression was systematically verified for the T cells we used, prior to each experiment, before activation and again after activation, right before use (Fig. 1A, Supplemental Fig. 1).

Trogocytosis differs from other mechanisms of intercellular Ag transfers, because it concerns entire portions of a donor cell’s plasma membrane and not individual molecules. Thus, trogocytosis has the potential of being a transfer of functional units from one cell to another. Thus far, we and others have demonstrated that acquired molecules can carry on their outward-oriented function (i.e., can act as a ligand and signal from the acquirer cell to another cell), but the possibility of a transferred receptor signaling inward has not been investigated in humans. In theory, this has always been a possibility on the condition that the originally functional membrane patch integrates properly within the plasma membrane of the acceptor cell and finds within its new cell a biochemical environment that it can reach and that is compatible with its functional requirements (e.g., signaling pathways). The data we presented in this study demonstrate that this is not only a possibility, but also a requirement for the well-described in vitro function of the inhibitory receptor ILT2.

The key findings of this article, as well as the trogocytosis-based mode of action of ILT2, are summarized in Fig. 7, which is only a schematic representation based on the data presented in Fig. 6. As depicted, during the first phase of the immune response, resting T cells do not express ILT2 and cannot acquire it from surrounding autologous monocytes for lack of Ag-independent trogocytic capability. It is possible that trogocytosis occurs during this phase, as reported in other systems. However, trogocytosis by resting cells is dependent on Ag recognition (5) and thus concerns molecules expressed by the presenting APC, not molecules expressed by bystander environmental cells, which do not present the selecting Ag. Immune regulation through molecules transferred from the stimulating APC may also happen at this time point but cannot be detected in our experimental system. At one point (day 4 in our case of allostimulation), activated Ag-specific T cells become capable of performing Ag nonspecific trogocytosis and acquire membranes from surrounding cells, including ILT2-containing membranes from autologous monocytes. The set of molecules involved in this type of acquisition is still unknown but clearly different from those used in Ag-specific trogocytosis by resting cells (9). Thus, through trogocytosis, activated T cells acquire multiple sensitivities from their microenvironment, which include sensitivity to inhibition by ILT2 ligands, as shown by our data. In the second phase of the response, T cells have become sensitive to a new array of immunomodulatory molecules. If these immunomodulatory molecules are present within the local environment, they may now signal to their transferred receptors and alter T cell behavior. In the case presented in this study, T cells became sensitive to ILT2 ligands, and because HLA-G was present within the local environment, these T cells stopped acting as effector cells. Thus, according to this model, the requirements for inhibition of T cells through ILT2 are: 1) Ag-selection followed by activation; 2) presence of ILT2-expressing monocytes; and 3) presence of an ILT2 ligand, HLA-G. To the best of our knowledge, it is the first time that transfers of sensitivity to immune modulatory molecules are shown and that this mechanism is proven crucial to an already acknowledged immune-regulation mechanism.

On a purely mechanistic standpoint, the data presented in this study show that membrane patches and molecules acquired by trogocytosis do not remain foreign patches, but integrate and, if possible, behave as would endogenously produced molecules. In our case, exogenously produced ILT2 was able to send an inhibitory signal strong enough to block the functions of the acceptor T cells, as would have endogenously produced ILT2. In our experimental configuration, donor and acceptor cells are both known to be able to signal through ILT2. It is therefore reasonable to postulate that the acquired patch integrated properly within the T cell plasma membrane and could use the compatible biochemical machinery of the acceptor cell to function. However, for other configurations in which donor and acquirer cells are biochemically very different, some signaling pathways might not be enabled, possibly leading to a partial or a lack of inward-oriented function of the acquired patch, even after integration of the transferred membrane. This limitation might be considered as a regulation mechanism for sensitivity transfers, allowing some but not all acquirer cells to acquire some but maybe not all sensitivities of the donor cell. Transfers of sensitivities would then be regulated at the cell-type level (trogocytic cell versus non trogocytic cell), molecular level (transferred versus not transferred molecules, integrated versus nonintegrated membrane patch), microenvironmental level (presence versus absence of a triggering ligand), and biochemical level (enabled versus disabled function of the transferred molecules). The possibility of inward signaling by acquired molecules through compatible biochemical pathways also raises two important but yet unresolved points: 1) if a signal is sent through a given membrane...
patch, will the consequences of this signal be different for the donor and the acceptor cells?; and 2) when considering only one signaling pathway and its consequences for a given cell, is it possible to broaden the range of triggering events for this signaling pathway through acquisition of new sensitivities?

From an immunological standpoint, our data show that trogocytosis is involved in a mechanism of in vitro immune regulation that is well known and may be important should it be confirmed in vivo. This is a key point, because if an immune regulation mechanism has been studied, agreed upon, and validated, so that it is regarded as a reliable fact, and if it is now proven that trogocytosis is required for this immune regulation mechanism, it means that trogocytosis might very well be involved in other immune mechanisms and thus may constitute a normal step and/or a general mechanism of immunity. This is first a problem: indeed, 1) if trogocytosis is a general mechanism of immune responses, the direct link among gene expression, phenotype, and function might be too much of a shortcut, and origin and traceability of molecules might become parameters (difficult ones) to seriously consider. Furthermore, 2) if trogocytosis is involved in or responsible for immunological phenomena currently studied or relied upon (such as ILT2-mediated inhibition of T cells), it means once more that the simplification of experimental systems by removal of bystander cells to obtain clean models might sometimes be just the wrong thing to do.

Yet, if intercellular transfers of cell-surface proteins, sensitivities, and functions constitute a generic mechanism in immunology, it means that there exists a level of powerful immune regulation that we hope is now clearly evidenced that is still vastly ignored and much of a shortcut, and origin and traceability of molecules might become parameters (difficult ones) to seriously consider. Furthermore, 2) if trogocytosis is involved in or responsible for immunological phenomena currently studied or relied upon (such as ILT2-mediated inhibition of T cells), it means once more that the simplification of experimental systems by removal of bystander cells to obtain clean models might sometimes be just the wrong thing to do.

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Disclosures

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References


