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RESEARCH ARTICLE

Different functional outcomes of intercellular membrane transfers to monocytes and T cells

Kiave-Yune HoWangYin · Estibaliz Alegre · Marina Daouya · Benoit Favier · Edgardo D. Carosella · Joel LeMaoult

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Abstract Trogocytosis is the uptake of membranes from one cell by another. Trogocytosis has been demonstrated for monocytes, B cells, T cells, and NK cells. The acquisition of the tolerogenic molecule HLA-G by T cells and NK cells makes them behave as regulatory cells. We investigated here whether HLA-G, which is expressed by tumor cells in vivo, could be acquired by monocytes and if this transfer could have functional consequences. We demonstrate that resting, and even more so, activated monocytes efficiently acquire membrane-bound HLA-G from HLA-G tumor cells by trogocytosis. However, we demonstrate that HLA-G quickly disappears from the surface of the monocytes in contrast to the HLA-G acquired by T cells. Consequently, HLA-G^{acq+} monocytes do not reliably inhibit the on-going proliferation of autologous activated T cells and do not inhibit their cytokine production. Thus, we show that the acquirer cell may control the functional outcome of trogocytosis.

Keywords HLA-G · Trogocytosis · Intercellular protein transfer · Immune regulation · Antigen presentation

K.-Y. HoWangYin and E. Alegre contributed equally to this work.

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K.-Y. HoWangYin · E. Alegre · M. Daouya · B. Favier · E. D. Carosella · J. LeMaoult
CEA, I2BM, Service de Recherches en Hemato-Immunologie, 75475 Paris, France

K.-Y. HoWangYin · M. Daouya · B. Favier · E. D. Carosella · J. LeMaoult
Institut Universitaire d'Hematologie, Hopital Saint Louis, 75475 Paris, France

E. Alegre
Department of Biochemistry, University Clinic of Navarra, Pamplona, Spain

J. LeMaoult (✉)
CEA-I2BM-SRHI, Hopital Saint Louis,
1 Avenue Claude Vellefaux, 75010 Paris, France
e-mail: Joel.LeMaoult@cea.fr

Introduction

Trogocytosis, reviewed in [1, 2], is a mechanism of fast, cell-to-cell contact-dependent uptake of membranes and associated molecules from one cell by another. During trogocytosis, all molecules contained within a certain membrane area are transferred, even if they are not involved in cell-cell cross-talk. What makes these transfers important is that the transferred membrane patches may temporarily endow the acceptor cell with some functions of the donor cells. Trogocytosis and its associated function have been extensively documented for the interaction between APC and T cells. Indeed (1) $CD8^+$ T cells that acquired their cognate MHC Class I^+ peptide ligands became susceptible to "fratricide" antigen-specific cytolysis [3, 4], (2) T cells that acquired HLA-DR and CD80 could stimulate resting T cells in an antigen-specific manner, and thus behave as APC themselves [5–7], (3) T cells could promote NK cell activity through trogocytosis of NKG2D and NKp46 ligands [8], and (4) $CD4^+$ T cells and NK cells that acquired HLA-G behaved as suppressor cells [9, 10]. Trogocytosis is not restricted to the interaction between APC and T cells. Many other types of cells may acquire membrane fragments [11–13]: For instance, immature DCs acquire antigens by trogocytosis from live cells for cross-presentation to T cells [14–16], and

monocytes or macrophages may also acquire membrane fragments from tumor cells [17].

HLA-G (reviewed in [18]) is characterized by a very low amount of polymorphism and an expression that is restricted to fetal trophoblast cells, adult epithelial thymic cells, cornea, erythroid precursors, and endothelial cell precursors. HLA-G can be expressed pathologically by non-rejected allografts, by lesion-infiltrating antigen presenting cells during inflammatory diseases, and by tumor tissues and/or their tumor infiltrating APC. HLA-G is further expressed by monocytes in multiple sclerosis, and by monocytes and T cells in viral infections. HLA-G is a potent tolerogenic molecule that strongly inhibits the function of immune cells. Indeed, HLA-G inhibits NK cell and cytotoxic T lymphocytes cytolytic activity, $CD4^+$ T cell alloproliferative responses, T cell (reviewed in [18]) and NK cell [10] ongoing proliferation, T cell and NK cell cytokine production [19, 20], and dendritic cell maturation [21]. HLA-G is also known to induce the differentiation of regulatory cells [21, 22]. HLA-G mediates its function through the interaction with three inhibitory receptors: ILT2 (CD85j/LILRB1) expressed by B cells, some T cells, some NK cells and all monocytes/dendritic cells [23], ILT4 (CD85d/LILRB2) expressed by myeloid cells [24], and KIR2DL4 (CD158d) [23–25] expressed by some peripheral and decidual NK cells.

We report here that HLA-G1 can be efficiently acquired from tumor cells by monocytes. In our system, non-activated monocytes, and much more so, activated monocytes acquired membrane-bound HLA-G from the surface of tumor cells. This process is mainly due to trogocytosis. HLA-G expression at the surface of monocytes is much shorter than at that of lymphocytes, and the suppressive function mediated by HLA-G is rapidly lost on monocytes. Thus, monocytes seem to be less able to act as regulatory cells than lymphocytes that had acquired HLA-G by trogocytosis [10]. These data demonstrate that the mechanisms involved in protein transfer and the function of trogocytosis are different from one type of cells to another.

Materials and methods

Cell isolation and cell lines

Blood was obtained from healthy volunteer donors under a protocol approved by the Institutional Review Board of the Saint Louis Hospital, Paris. Peripheral-blood mononuclear cells (PBMCs) were isolated on Histopaque® 1077 density gradient (Sigma).

For all purification steps with beads, Fc receptors on PBMCs were blocked using 20 μg/ml human IgG (Sigma) for 30 min. For isolation of monocytes, PBMCs were labeled with 20 μg/ml anti-CD14 and then positively separated using GAM-coated magnetic beads according to the manufacturer's specifications (Ademtech, France). Alternatively, monocytes were also purified by adherence on plastic.

The adherent melanoma cell line M8 transfected with pcDNA3.1 (Invitrogen) alone (M8-pcDNA) or with pcDNA3.1 containing HLA-G1 cDNA (M8-HLA-G1), and the lymphoblastoid LCL-721.221 cells (LCL; ATCC, Rockville, MD) transfected with pRc/RSV (Invitrogen) alone (LCL-RSV) or with pRc/RSV containing HLA-G1 cDNA (LCL-HLA-G1) have been previously described [9], as have M8 cells transfected with the HLA-G1-EGFP fusion protein (M8-HLA-G1-EGFP) [10].

All cells were cultured in RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine, 1 μg/ml gentamicin and fungizone (Sigma), and 10% heat-inactivated FCS.

Cell activation

When indicated, monocytes were activated with 100 ng/ml of lipopolysaccharide (LPS) (Sigma).

Prior to use in trogocytosis assays, T cells were activated for 48 h with PHA-L (2 μg/ml, Sigma) and subsequently by a 24- to 48-h culture in medium supplemented with 100 IU IL2 (Sigma), as described in [9].

Proliferating T cells used in functional assays were obtained by a 48-h activation by PHA-L.

Antibodies and flow cytometry

From Exbio, Prague, Czech Republic: purified anti-CD14, blocking anti-HLA-G 87G Fab, non-blocking anti-HLA-G MemG09, biotinylated anti-HLA-G 87G, PE-Dy-647 and PE-Dy-590 conjugated anti-CD14. From Beckman Coulter: FITC-conjugated anti-CD14; from BD Biosciences: purified and PE-conjugated anti-ILT2 (clone GHI/75), purified anti-HLA-G (4H84). From Invitrogen: streptavidin Alexa 405, Goat-Anti-Mouse (GAM) Alexa Fluor® 647. Labeling of the plasma membrane of monocytes was performed using the PKH67 Green Fluorescent Cell Linker Mini kit (Sigma) or the PKH26 Red Fluorescent Cell Linker kit (Sigma) according to the manufacturer's specifications.

Prior to staining for flow cytometry, Fc receptors were blocked in 25% human serum supplemented with 20 μg/ml human IgG (Sigma). Appropriate isotypic controls were systematically used to evaluate non-specific binding. For intracellular staining, cells were fixed 10 min with 3% paraformaldehyde and then permeabilized with PBS1X/3% BSA/0.3% saponin.

Flow cytometry analyses were performed on an Epics XL Cytometer (Beckman Coulter) using EXPO32 software (Beckman Coulter).

Confocal microscopy

Monocytes were incubated with PKH26-labeled M8-HLA-G1-EGFP cells at 37°C for 30 min. Staining was performed with biotinylated anti-HLA-G (87G) followed by streptavidin Alexa 405 and with anti-CD14 followed by GAM Alexa Fluor® 647 according to the flow cytometry staining protocol. Samples were then left adhered on poly-L-lysine coated slides for 5 min at 37°C, and fixed for 10 min with 3% paraformaldehyde (PFA). Samples were finally mounted in a VECTASHIELD mounting medium with DAPI (Clinisciences, Paris, France) and analyzed using a Carl Zeiss LSM 510 confocal microscope (Zeiss, Germany).

Trogocytosis assays

Trogocytosis assays have been described in [9]. Briefly, purified monocytes or activated PBMCs were co-cultured at 37°C in 5% CO_2 for 30 min with tumor cells at a 1:1 ratio, to a total concentration of 10^6–10^7 cells per ml. Following this co-incubation, the presence of membranes (trogocytosis process) from tumor cells was sought on T cells or monocytes. Prevention of cell-cell contact was performed using a Transwell culture system (Greiner Bio-One, Kremsmünster, Austria) [9]. After trogocytosis, cells were placed on ice, and all further steps were performed on ice. Throughout the manuscript, T cells or monocytes co-incubated with HLA-G-positive tumor cells that acquired HLA-G by trogocytosis are called HLA-G^{acq+} T cells or HLA-G^{acq+} monocytes, respectively. Similarly, T cells or monocytes co-incubated with HLA-G negative tumor cells are called HLA-G^{acq-} T cells or HLA-G^{acq-} monocytes after trogocytosis assays.

Blocking of HLA-G:ILT2 interaction

Cells were first incubated 30 min in 25% human serum supplemented with 20 µg/ml human IgG to block Fc receptors. Blocking anti-HLA-G 87G Fab or blocking anti-ILT2 antibodies, or their isotypic controls, were then added at a concentration of 10 µg/ml. Cells were then used in co-incubations, and all blocking conditions were maintained during all the steps of the experiments.

Inhibition of on-going proliferation of PBMC by HLA-G^{acq+} cells

The 10^5 already proliferating PHA-L activated PBMCs were co-cultured for 18 h with HLA-$G1^{acq+}$ monocytes or HLA-$G1^{acq-}$ monocytes in 200 µl of culture medium supplemented with 1 µCi of tritiated thymidine (^{3}H-thymidine, Amersham Biosciences).

Proliferation of 10^5 autologous HLA-G^{acq+} T cells or HLA-G^{acq-} T cells was taken as positive and negative control, respectively, of inhibition of on-going proliferation of T cells by HLA-G acquired by trogocytosis on T cells.

The proliferation of PBMC was measured by ^{3}H-thymidine incorporation in an 18-h period, using 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.

Cytokine assays

The 10^5 PHA-L-activated PBMCs were re-stimulated with 10 µg/ml of PHA-L. Re-stimulated PBMCs were then co-incubated in a volume of 100 µl with HLA-$G1^{acq+}$ or HLA-$G1^{acq-}$ monocytes. Brefeldin A (Sigma-Aldrich, 10 µg/ml), an inhibitor of intracellular protein transport, was added at this time, and cells were incubated 4 or 18 h at 37°C, 5% CO_2. The IFN-G expression was analyzed by flow cytometry using the FITC-conjugated anti-IFN-G and PE-Dy590-conjugated anti-CD3, according to the intracellular staining protocol described earlier, and the protocol described in [26]. Samples were run as duplicates. Histograms were analyzed by calculating the percentage of IFN-G-producing T cells in the co-culture.

Statistical analyses

Data are presented as mean ± standard deviation (SD). Student's t test was used, and a P value less than 0.05 was taken to be significant. For figures showing representative experiments, error bars represent SD of triplicates.

Results

Monocytes acquire HLA-G from tumor cells

In order to verify if HLA-G expressed by a tumor cell could be acquired by monocytes and displayed at their surface, we co-incubated freshly isolated monocytes with HLA-G1-transfected melanoma cells (M8-HLA-G1) or HLA-G1-transfected lymphoblastoid cells (LCL-HLA-G1) and their HLA-G1-negative counterparts (M8-pcDNA and LCL-RSV, respectively) for 30 min or less.

Figure 1a shows that monocytes did not display HLA-G prior to co-incubation with tumor lines or after a 30-min co-incubation with HLA-G-negative M8-pcDNA or LCL-RSV cells. However, after a 30-min co-incubation with HLA-G-positive M8-HLA-G1 or LCL-HLA-G1 cells, 26% or more of monocytes displayed HLA-G at their surface.

In order to prove that in our system cell-surface HLA-G1 that was detected on monocytes after co-incubation

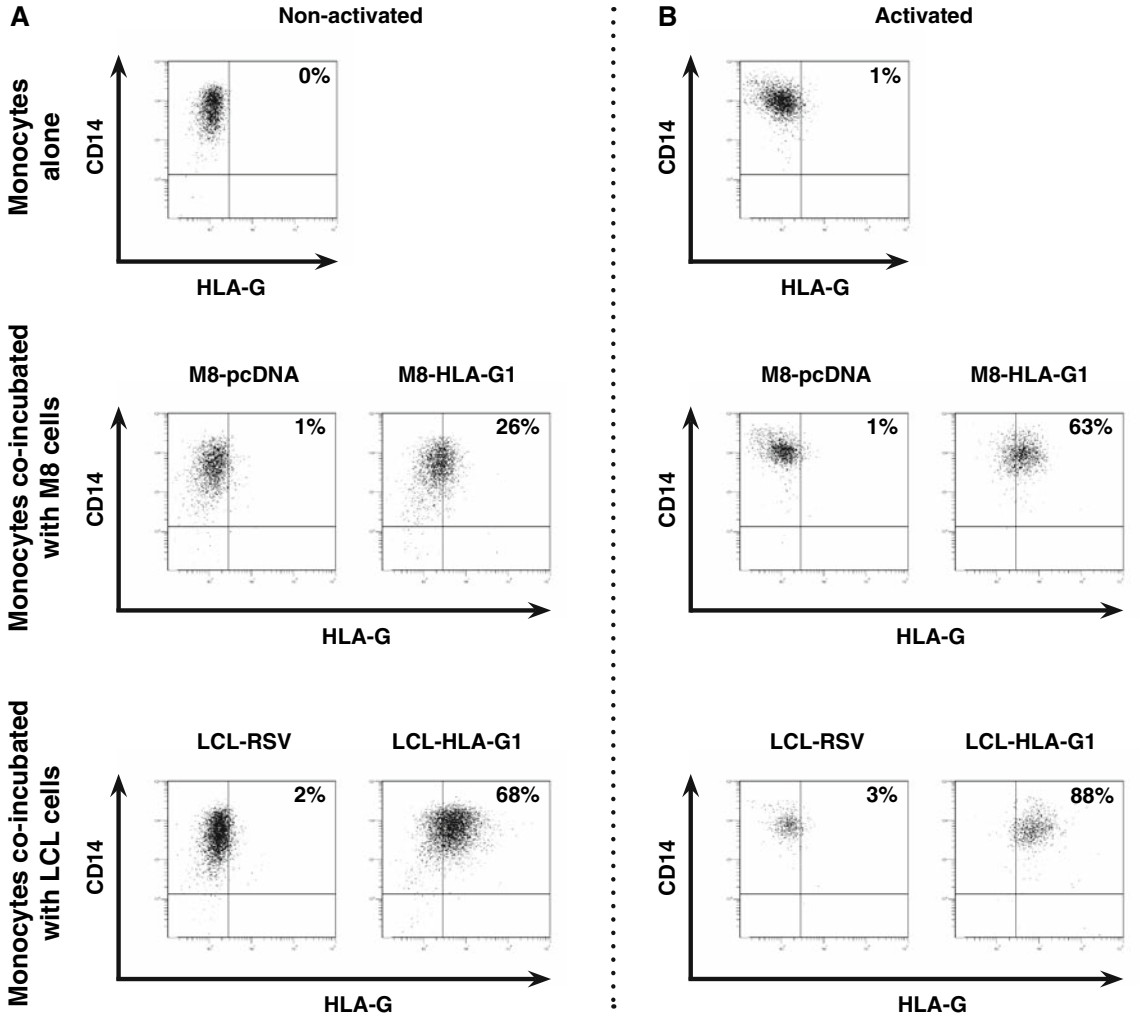


Fig. 1 HLA-G cell-surface display by monocytes prior to (monocytes alone) and after a 30-min co-incubation with HLA-G-positive M8-HLA-G1 and LCL-HLA-G1 tumor cells and their HLA-G negative M8-pcDNA and LCL-RSV counterparts. **a** Non-activated monocytes. **b** Monocytes activated 3 days with LPS. Experiment shown is representative of three

with HLA-G-positive cells was not endogenously produced, we performed an HLA-G intracellular staining of the monocytes at the time of co-incubation. No intracellular HLA-G was detected (Supplemental Figure 1). Given the short kinetics of our experiments (30 min or less), this indicates that neo-expression was very unlikely.

Trogocytosis capability was shown to be increased by activation in various experimental settings [9, 10]. In order to find out if monocytes also required activation to take up proteins from their surroundings, we investigated the HLA-G capture of monocytes activated 3 days with LPS. Figure 1b shows that even if monocytes could acquire HLA-G without any particular activation, HLA-G uptake was significantly enhanced by activation. Indeed, 68% of non-activated versus 88% of activated monocytes acquired HLA-G from LCL-HLA-G1 cells, and 26% of non-activated monocytes versus 63% of activated monocytes acquired HLA-G from M8-HLA-G1 cells.

In the next set of experiments, we investigated whether activated monocytes became HLA-G-positive through a trogocytosis mechanism.

HLA-G is acquired from tumor cells by trogocytosis

Trogocytosis is characterized by (1) fast kinetics, (2) transfers of membrane patches and (3) membrane-bound molecules, (4) cell-to-cell contact dependence, and (5) a short half-life of the acquired molecules. We investigated these parameters to prove that HLA-G transferred from M8-HLA-G1 cells to monocytes by trogocytosis.

1. The fast kinetics of HLA-G acquisition by monocytes is illustrated in Fig. 2: 30 min of co-incubation was

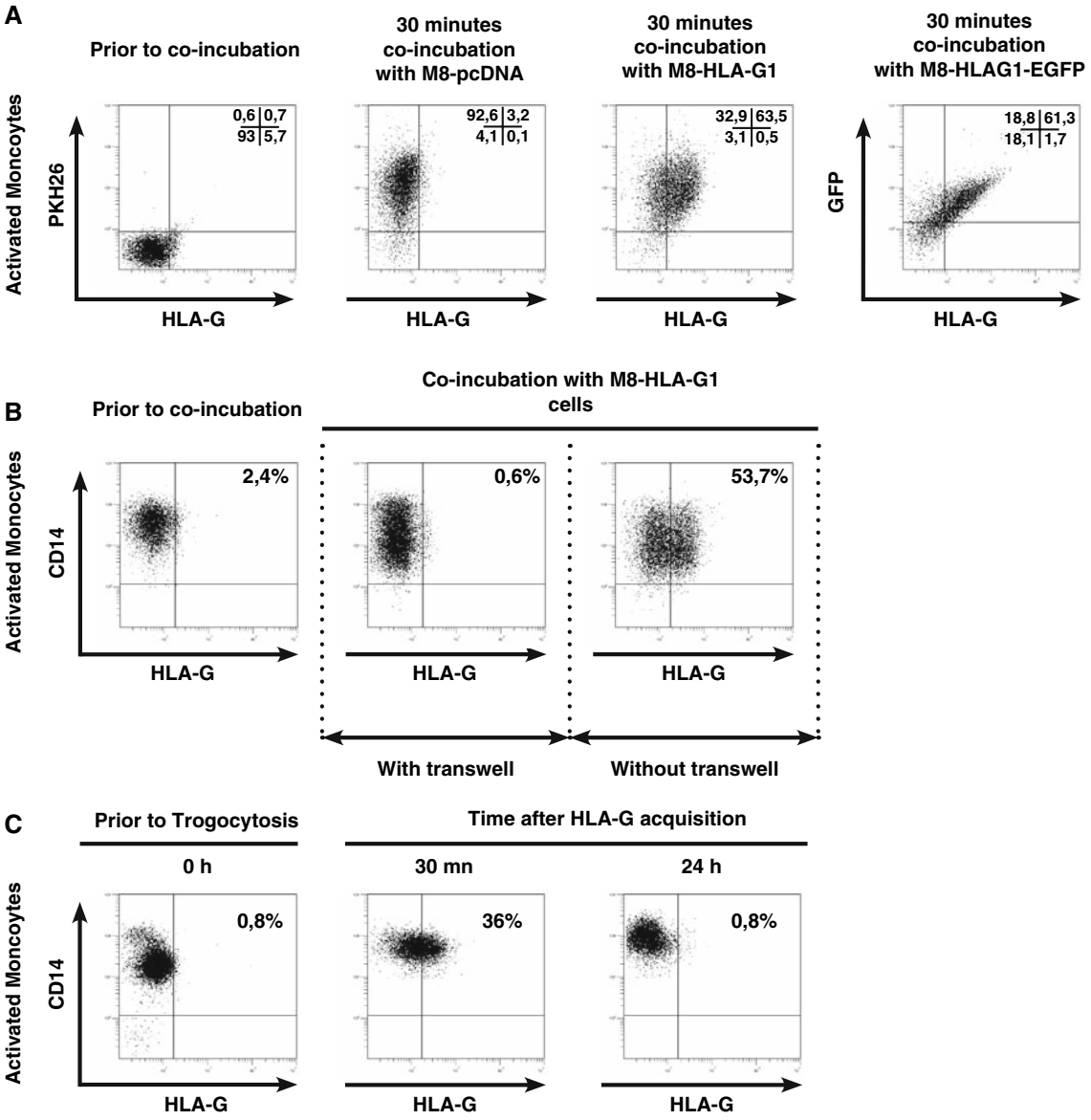


Fig. 2 HLA-G display by monocytes is due to trogocytosis. **a** Monocytes display full-length HLA-G1 at their surface along with membranes from HLA-G1^{+} APC. Flow cytometry was performed on monocytes prior to and after co-incubation with PKH26-labeled M8-pcDNA, or PKH26-labeled M8-HLA-G1, or M8-HLA-G1-EGFP cells. Expression of PKH, HLA-G1, and GFP by monocytes was assessed by flow cytometry. Results are expressed as percentage of cells. **b** Cell-to-cell contact between monocytes and M8 HLA-G1 is required for HLA-G1 transfer on monocytes. Monocytes and M8-HLAG1 cells were co-incubated for 30 min together (no Transwell) or separated by a semi-permeable membrane (Transwell) before analysis of monocyte HLA-G1 cell-surface expression. **c** HLA-G1 expression by monocytes is transitory. Monocytes were co-incubated for 30 min with M8-HLA-G1 and then purified. Acquisition of HLA-G1 was assessed by flow cytometry 30 min or 24 h after the beginning of co-incubation

sufficient to observe HLA-G transfer to 63% of activated monocytes.

2. Figure 2a also shows that when monocytes were co-incubated with M8-HLA-G1 cells whose membrane had been labeled with a lipophilic dye (PKH26); they became positive for PKH26, which shows they had acquired membrane from M8-HLA-G1 cells. Lastly, Fig. 2a shows a correlation between monocyte HLA-G positivity and PKH positivity after co-incubation with M8-HLA-G1 cells. This shows that HLA-G was most likely included within the membranes that transferred from M8-HLA-G1 cells to LPS-activated monocytes.
3. To prove that HLA-G1 displayed by monocytes was intact and not a molecule shed from the surface of M8-HLA-G1 cells, we used as donors cells M8-HLA-G1-EGFP cells that expressed an HLA-G1-EGFP fusion

protein constituted of an EGFP bound to the intracellular part of HLA-G1. Figure 2a shows that GFP fluorescence of the intracellular part transferred along with HLA-G1 extracellular domains, which indicates that it was the entire membrane-bound HLA-G that transferred and not a shed molecule.

4. Figure 2b shows that cell-to-cell contact is required for HLA-G display by monocytes after co-incubation with M8-HLA-G1 cells. Indeed, separating M8-HLA-G1 and monocytes by a semi-permeable membrane of a transwell unit prevented it.
5. Figure 2c shows that HLA-G acquisition is temporary: acquired HLA-G has a limited lifespan at the surface of monocytes and has completely disappeared 24 h after acquisition from tumor cells. This also shows that in our system, monocytes never expressed their own membrane-bound HLA-G1.

A confocal visualization of HLA-G trogocytosis by monocytes with a large membrane patch of M8-HLA-G1-EGFP origin on a monocyte is shown in Fig. 3a. This image also shows the co-localization of the fluorescence characteristic of the intracellular part of HLA-G (GFP), the extracellular part of HLA-G, and the M8-HLA-G1 membrane in between (PKH).

Thus, activated monocytes acquire membrane-bound HLA-G from tumor cells through membrane transfers. Yet, this is not sufficient to say they acquire HLA-G and membranes by trogocytosis. Indeed, monocytes have been shown to acquire membranes from other cells through nanotubes. Nanotubes can either be actin-driven protrusions extending from one cell to another [27] or tethers that form when two cells dissociate after cell-to-cell interaction

Fig. 4 Turnover of HLA-G at the surface of acquirer monocytes. ▶ **a** The persistence of the fluorescence associated with HLA-G and PKH26 of PKH26-labeled M8-HLA-G1 origin was evaluated by flow cytometry on HLA-G^{acq+} monocytes prior to and 30 min and 24 h after acquisition. **b** The persistence of the fluorescence associated with HLA-G and GFP of M8-HLA-G1-EGFP origin was evaluated by flow cytometry on HLA-G^{acq+} monocytes prior to and 30 min and 24 h after acquisition. **c** Comparative evaluation of the kinetics of HLA-G disappearance from the surface of HLA-G^{acq+} monocytes and autologous HLA-G^{acq+} T cells. The percentage of HLA-G-positive cells and the mean fluorescence intensity expressed as percentage of initial intensity are shown at various time points. Experiment shown is representative of three

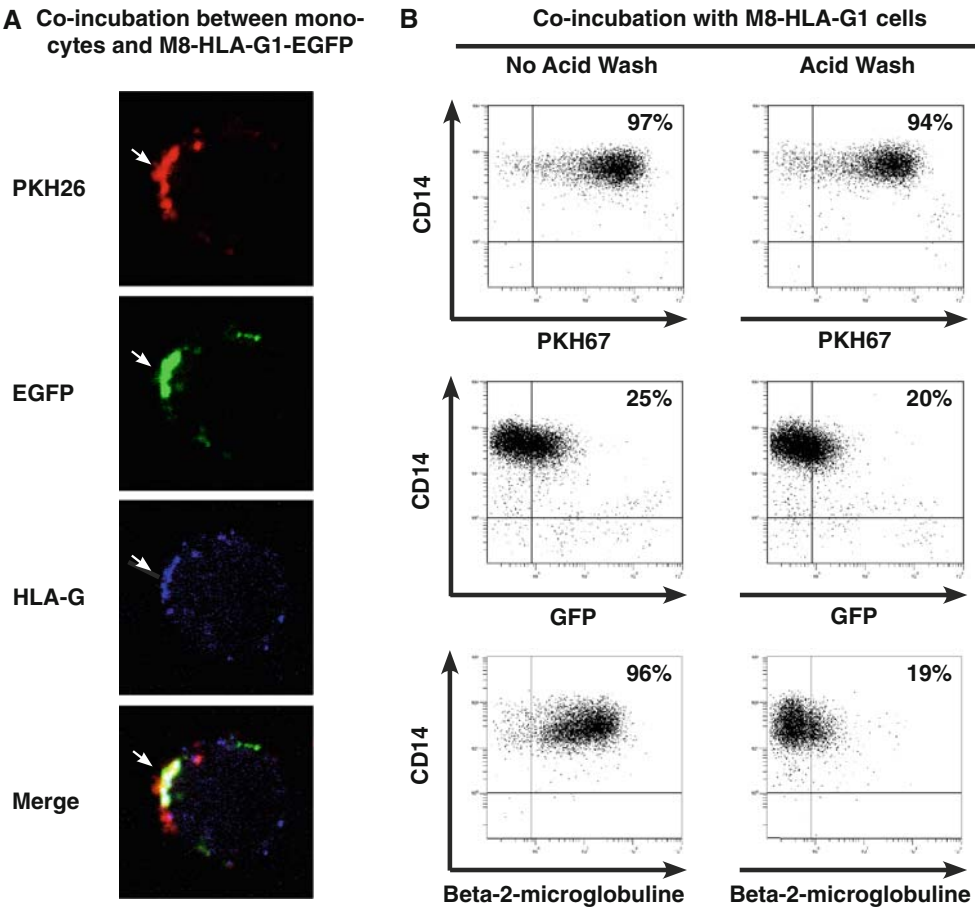
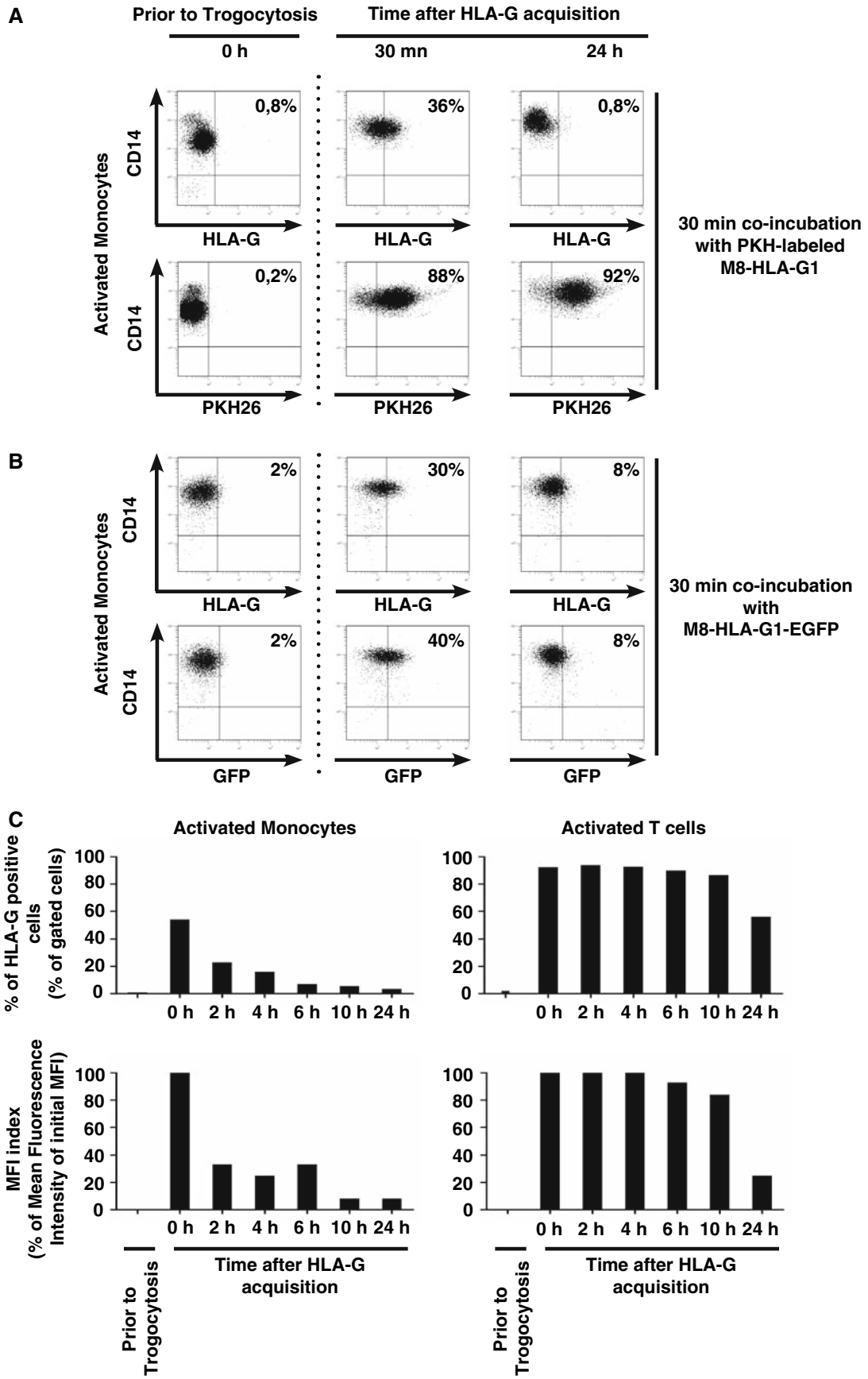


Fig. 3 Transferred HLA-G localizes within acid-resistant large membrane patches of donor origin. **a** HLA-G localizes within large membrane patches of donor origin. Confocal microscopy was performed on monocytes co-incubated with PKH26-labeled M8-HLA-G1-EGFP. *Blue* HLA-G staining (DAPI), *green* GFP, *red* M8-HLA-G1 membranes (PKH26). Data show the co-localization of M8-HLA-G1 membranes and HLA-G on monocytes, indicative of a M8-HLA-G1 origin of HLA-G. *Arrows* point to areas of interest. **b** Acid wash does not remove the acquired membrane. Monocytes were treated or not by acid wash after co-incubation with PKH67-labeled M8-HLA-G1 cells or M8-HLA-G1-EGFP cells. PKH67 and GFP staining was assessed by flow cytometry. Beta-2-microglobulin staining was used as a positive control of the acid wash efficiency. Experiment shown is representative of three


A
Prior to Trogocytosis
Time after HLA-G acquisition
0 h
30 mn
24 h
Activated Monocytes
CD14
HLA-G
0,8%
36%
0,8%
0,2%
88%
92%
PKH26
30 min co-incubation with PKH-labeled M8-HLA-G1
B
2%
30%
8%
2%
40%
8%
GFP
30 min co-incubation with M8-HLA-G1-EGFP
C
Activated Monocytes
Activated T cells
% of HLA-G positive cells (% of gated cells)
MFI index (% of Mean Fluorescence Intensity of initial MFI)
0 h 2 h 4 h 6 h 10 h 24 h
Prior to Trogocytosis
Time after HLA-G acquisition

[28]. In this case, membranes from the donor cell remain affixed onto the cell surface of the acceptor cell and can be removed by a mild acid wash treatment [29]. In order to address this issue, we co-incubated monocytes either with PKH-labeled M8-HLA-G1 or with M8-HLA-G1-EGFP, and then purified them. Figure 3b shows that only 4.78% ± 10.04 ($n = 3$) of the PKH-labeled membranes of M8-HLA-G1 origin were removed by mild acid treatment. Similarly, only 12.7% ± 11.2 ($n = 3$) of GFP fluorescence of M8-HLA-G1-EGFP origin were removed. By contrast, mild acid treatment successfully removed 83.9% ± 11.6 ($n = 3$) of non-covalently bound beta-2-microglobulin. These data suggest that nanotubes were not involved in HLA-G transfer in this experimental system. Our results do not rule out HLA-G transfer from M8-HLA-G1 cells by nanotubes, but they mean that nanotubes are not the main mechanism of transfer in this particular case.

HLA-G quickly disappears from the surface of monocytes

We have demonstrated that HLA-G was acquired by trogocytosis and that monocytes only transiently expressed this molecule. Since monocytes have a high membrane recycling rate [30], we investigated the turnover of the acquired molecule at their surface.

To this aim, we incubated monocytes with PKH-labeled tumor cells (M8-HLA-G1) and then purified the monocytes. Figure 4a shows that after 30 min, monocytes were, as expected, HLA-G-positive and PKH-positive. After 24 h, all the HLA-G had disappeared from the surface of the monocytes, as we have already shown in Fig. 2c, whereas nearly all the PKH staining remained. This could be due to shedding of the HLA-G molecule or to its internalization. Indeed, once internalized, HLA-G is no longer accessible to antibodies, whereas the fluorescence associated with PKH-labeled membrane is still observable.

In order to verify that the disappearance of the acquired HLA-G molecules was not due to their shedding, we performed the same experiment using M8-HLA-G1-EGFP as donor cells. Figure 4b shows that the GFP fluorescence also disappeared after 24 h, ruling out shedding of acquired HLA-G, which concerns the extracellular part of HLA-G but would have left EGFP intact. These data strongly suggest that the acquired membranes had been internalized, processed, and their protein contents degraded, including HLA-G and GFP.

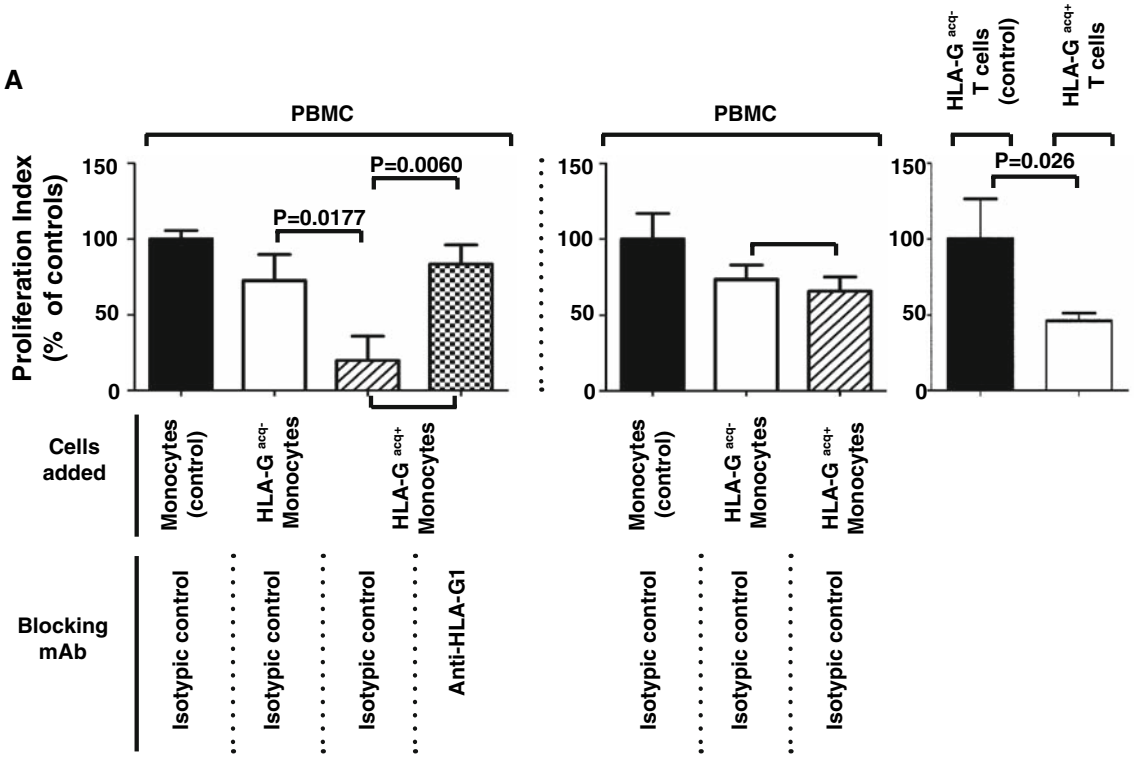
We next investigated the turnover of acquired HLA-G at the surface of monocytes and compared it to that of HLA-G acquired by trogocytosis from the same tumor cells by autologous T cells. Figure 4c shows that the lifespan of HLA-G is much shorter at the surface of monocytes than that of lymphoyctes. Indeed, 10H after trogocytosis, less than 10% of T cells had lost HLA-G expression and the MFI index of the T lymphoyctes had diminished by only 20%, whereas, after 2 h, more than 50% of the originally HLA-G positive monocytes had lost HLA-G expression, and the MFI of the remaining HLA-G positive monocytes had decreased by 70%. Ultimately, HLA-G staining was lost for the two cell types 24 h after acquisition. Thus, molecules acquired by trogocytosis disappeared more quickly at the surface of monocytes than on T cells.

Function of HLA-G^{acq+} monocytes on the proliferation of PBMC

We investigated whether, as we described for T cells and NK cells, HLA-G^{acq+} monocytes had gained the capability to act as regulatory cells and could stop the on-going proliferation of PHA-activated PBMC through the HLA-G molecules they had acquired. For this purpose, we generated HLA-G^{acq+} monocytes and HLA-G^{acq-} monocytes by co-incubation with M8-HLA-G1 and M8-pcDNA cells, respectively, purified them, and added them to a culture of PHA-activated and already proliferating autologous PBMC. We then compared the effect of HLA-G^{acq-} monocytes and HLA-G^{acq+} monocytes on the proliferation of activated PBMC. Figure 5a shows the two types of results we obtained. In two cases (shown on the left), the on-going proliferation of PHA-activated PBMC could clearly be inhibited by the addition of HLA-G^{acq+} monocytes, whereas the addition of HLA-G^{acq-} monocytes had almost no effect. Thus, for these two experiments, the trogocytic acquisition of HLA-G-containing membranes from tumor cells by activated monocytes made them function as regulatory cells. Furthermore, this regulatory function was abrogated by masking HLA-G at their surface, which demonstrates that they mediated their regulatory function directly through acquired HLA-G. This is exactly what had been observed for T and NK cells [9, 10, 30].

However, these results were hardly representative (2 out of 14 experiments): in the majority of the experiments (shown on the right), the addition of HLA-G^{acq+} monocytes to proliferating autologous PBMC had no effect. This was not due to a lack of HLA-G function. HLA-G acquired from the same tumor cells by autologous activated T cells inhibited T cell on-going proliferation, as previously reported [9]. Thus, we showed that HLA-G acquired by T cells by trogocytosis is capable of inhibiting T cell proliferation, whereas HLA-G acquired by monocytes by trogocytosis is not.

Figure 5b shows the amount of HLA-G acquired by trogocytosis on monocytes related to the inhibitory function of HLA-G^{acq+} monocytes in the proliferation assays. No significant difference of percentage of HLA-G positive



B

HLA-G inhibition	YES n=2		NO n=12
% of HLA-G positive cells	82,6	11,7	41.57 ± 15.93
Specific Mean Fluorescence Intensity	6,4	1,5	3.15 ± 1.94

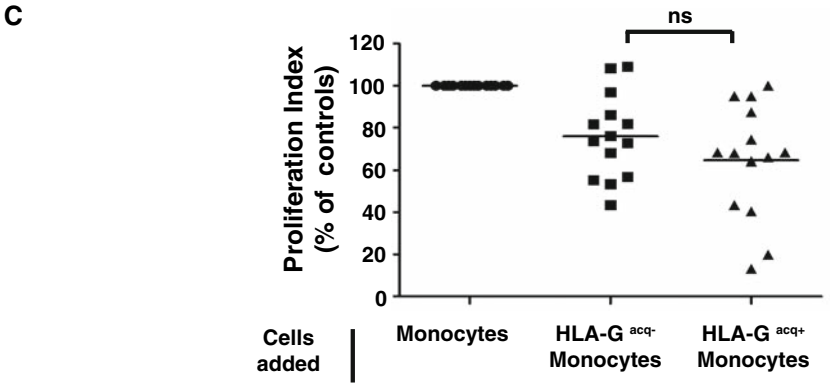


Fig. 5 Inhibition of on-going proliferation by HLA-G^{acq+} monocytes. **a** Thymidine incorporation for PBMC co-incubated with monocytes, HLA-G^{acq-} monocytes, or HLA-G^{acq+} monocytes with or without anti-HLA-G1. The two individual experiments shown are representative of the two outcomes observed. The proliferation inhibition of PHA-L-activated T cells by trogocytosed HLA-G (HLA-G^{acq-} T cells versus HLA-G^{acq+} T cells, bar graph) is shown as a control of HLA-G capability to function after transfer, as published in [9, 10]. Results shown are expressed as percentage of proliferation of PBMC compared to controls. **b** *Table* indicating the amounts of acquired HLA-G on the monocytes used in the 14 proliferation assays. **c** Dot plot of 14 independent experiments. Results are expressed in percentage of the proliferation of PBMC incubated with non-manipulated monocytes

cells or specific mean fluorescence intensity was observed between HLA-G^{acq+} monocytes that induced an inhibitory function or HLA-G^{acq+} monocytes that had no effect on the on-going proliferation of activated PBMC. Thus, the function of HLA-G^{acq+} monocytes is not linked with the initial amount of HLA-G at the surface of the monocytes.

Figure 5c shows the mean of the percentage of PBMC proliferation compared to controls of 14 independent

experiments when monocytes or HLA-G^{acq+} monocytes or HLA-G^{acq-} monocytes were added to the proliferation of activated autologous PBMC. Overall, no significant difference was observed between the addition of HLA-G^{acq+} monocytes and HLA-G^{acq-} monocytes on the proliferation of autologous activated PBMC.

Thus, HLA-G^{acq+} monocytes, even though they express HLA-G briefly, do not reliably inhibit the on-going proliferation of autologous PBMC.

Function of HLA-G^{acq+} monocytes on the cytokines production of T cells

It has already been reported that HLA-G inhibits the IFN-G production of lymphocytes [19, 26]. Therefore, even though HLA-G acquired by trogocytosis on monocytes had no effect on the on-going proliferation of already activated PBMCs, we investigated whether HLA-G^{acq+} monocytes could inhibit the IFN-G production of autologous T cells.

We generated HLA-G^{acq-} and HLA-G^{acq+} monocytes, purified them, and co-cultured them with activated PBMC. We then evaluated IFN-G production by T cells by flow cytometry.

Figure 6 shows the relative percentage of IFN-G-producing T cells co-cultured with monocytes, HLA-G^{acq-} monocytes, HLA-G^{acq+} monocytes, or HLA-G^{acq+} monocytes with an anti-HLA-G. M8-pcDNA and M8-HLA-G1 cells were used as negative and positive controls, respectively, of the inhibition of the T cell IFN-G production by HLA-G. After 4 h or after 18 h of co-culture, the results obtained were similar. Results show that IFN-G production of T cells was not inhibited by HLA-G^{acq+} monocytes or HLA-G^{acq-} monocytes. By contrast, M8-HLA-G1 cells efficiently inhibited the IFN-G production of T cells, as compared with M8-pcDNA cells. Therefore, HLA-G acquired by monocytes by trogocytosis is not able to inhibit T cell IFN-G production.

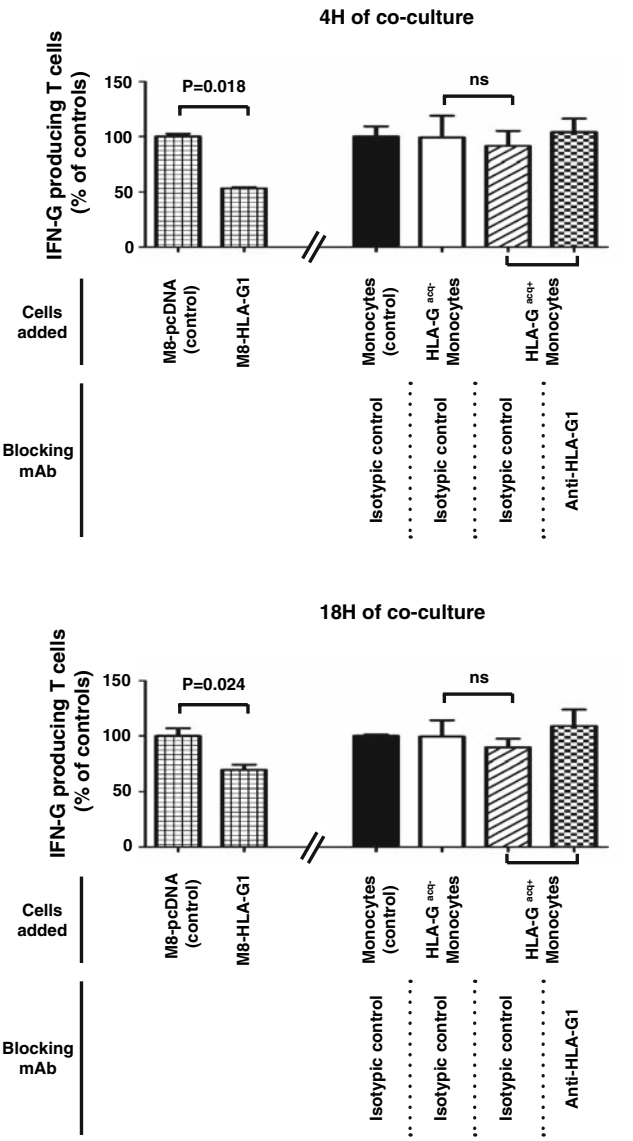


Fig. 6 IFN-G production by T cells. T cells were co-incubated with monocytes, HLA-G^{acq-} monocytes, or HLA-G^{acq+} monocytes, and T cell IFN-G production was measured by flow cytometry. M8-pcDNA and M8-HLA-G1 were used to show the capability of HLA-G to inhibit T cell IFN-G production. Results are expressed as percentage of cytokine production of T cells compared with non-manipulated monocytes. Experiment shown is representative of three

Discussion

In this study, we addressed the question of the functional outcome of trogocytosis on different cells. For this, we compared the functional outcome of HLA-G trogocytic acquisition from tumor cells by autologous monocytes and T cells.

The results presented here show that T cells and monocytes both efficiently acquire the tolerogenic molecule HLA-G1 from HLA-G-expressing tumor cells by trogocytosis. It is known that HLA-G may be endogenously expressed by some monocytes, as a membrane-bound or as a soluble isoform, especially in pathological conditions. To avoid any ambiguity as to where the HLA-G we studied came from, we used monocytes that did not express surface or intracellular HLA-G. It has also been reported that HLA-G expression could be induced in monocytes by treatment with cytokines such as IL-10, IFN-gamma, and IFN-beta. We did not use these stimulations, only LPS-activated monocytes, therefore ruling out any potential effect of such stimulations. Finally, in all our experiments, we compared the function of HLA-G^{acq+} monocytes with HLA-G^{acq-} monocytes to ensure no endogenous HLA-G was produced and interfered with the functional studies of acquired HLA-G.

Our data demonstrate that the acquisition of HLA-G1 by monocytes is achieved through trogocytosis and that this transfer is greatly enhanced by monocyte activation, although it does not require it. Moreover, blocking studies with an anti-HLA-G or an anti-ILT2 showed that HLA-G trogocytosis was not mediated through HLA-G or its receptor ILT2 (Supplemental Figure 2). We further found that HLA-G remained a longer time at the surface of T cells than at that of monocytes and that the suppressive function mediated by HLA-G is rapidly lost on monocytes.

Trogocytosis by monocytes did not differ from trogocytosis by other cells, described in the context of the interaction between T cells or NK cells and APC and tumor cells. Yet, significant differences were observed: whereas the trogocytic capability of T cells and NK cells has been associated with activation, we demonstrated here that non-activated monocytes could acquire membranes from HLA-G-expressing tumor cells, albeit less efficiently than activated monocytes. Furthermore, we showed that HLA-G taken from the same sources remained a longer time at the surface of T cells than on that of monocytes. The fast removal of HLA-G might be due to a faster internalization and degradation of HLA-G by monocytes.

Several articles have already reported the importance of internalization and endocytosis after trogocytic transfer. For T cells, MHC class I molecules acquired from APC are internalized with the TCR they bind [31]. NK cells could internalize also HLA-C molecules acquired from target cells [32]. This is also the case for monocytes, where trogocytosis mediated by antibodies leads also to the internalization of the antigens acquired [17]. It is thus possible that both trogocytosis by non-activated monocytes and the short lifespan of the acquired molecules at the monocyte surface are linked to the primary function of monocytes, which is to uptake, process, and present antigens from their soluble or cellular environment. In this context, as postulated for immature dendritic cells [14], trogocytosis might constitute a mechanism for monocytes to acquire membrane-bound molecules from living cells, and not only apoptotic ones. Others have investigated the potential impact of antigen acquisition by dendritic cells, and it was shown that in the context of allotransplantation, recipient APCs might "acquire and present intact donor MHC class I molecules to direct pathway $CD8^+$ T cells, and simultaneously present internalized and processed donor MHC molecules as peptides to $CD4^+$ T cells with indirect anti-donor allo-specificity," and so stimulate direct anti-donor allo-immune response. This mechanism was named semi-direct allo-recognition by the authors [15, 33]. Our data are in agreement with these findings on antigen acquisition by monocytes and do not contradict the semi-direct presentation hypothesis. Yet, our data show for HLA-G, which is known to be internalized slowly compared to other HLA molecules, a fast disappearance from the cell surface of the monocytes. This might not be compatible with the notion that immature dendritic cells take up allogeneic antigens, and then have the time to migrate to lymph nodes and present these intact acquired antigens to T cells. Moreover, due to rapid membrane turnover, it was shown that the half-life of the antigens presented at the surface of monocytes is much shorter than their half-life when presented by dendritic cells [30].

In any case, it is clear that through membrane transfers, many more molecules than just immunogenic HLA molecules are transferred, some of which do not need a migration to lymph nodes to act. This is the actual context of this study, where the transferred molecules may act in situ, in the vicinity of the antigen-providing cells.

In the case presented here, the transferred antigen is HLA-G. HLA-G is an inhibitory molecule that can inhibit the alloproliferative T cell response [22, 34], the ongoing proliferation of T cells [35], and the differentiation/activation of monocytes [21]. When transferred from APC to T cells and from tumor cells to NK cells, HLA-G retains its inhibitory function and makes the immune effector cells act as regulatory cells capable of inhibiting each other as well as other cells [9, 10]. We demonstrated that monocytes also acquire membrane-bound HLA-G by trogocytosis, but due to a quick turnover, HLA-G acquired by trogocytosis has no inhibitory effect on the proliferation of autologous activated T cells or on the production of the pro-inflammatory cytokine IFN-G. In two cases (out of 14), we nevertheless found an inhibitory function of $HLA\text{-}G^{acq+}$ monocytes on the proliferation of autologous activated T cells. This inhibition was not due to a greater amount of HLA-G at the surface of the monocytes. Our hypothesis is that "trogocytosed" HLA-G, in these two cases, remained a longer time at the surface of the monocytes, whereas in the samples where no inhibition was observed, it was internalized too quickly.

Thus, the function and consequence of trogocytosis in the case of monocytes might be different from other systems already described [9, 15]. Indeed, compared to what has been published for lymphocytes, trogocytic monocytes acquire membranes more easily and more efficiently than lymphocytes, but on the other hand, acquired molecules have a shorter turnover at the surface of a monocyte than at the surface of a lymphocyte. Which, then, of the monocytes or the lymphocytes will best use intact molecules acquired by trogocytosis is unknown. Given that APCs are known to share membranes with lymphocytes by trogocytosis, one can also wonder if the only fate of membranes acquired by monocytes is internalization and processing, or whether monocytes may act as collectors of local membranes, which they might temporarily present to T cells prior to internalization, as already postulated, or, alternatively, transfer again to nearby immune cells.

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