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**Supplementary Data**

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Antigen-Specific Transfer of Functional Programmed Death Ligand 1 from Human APCs onto CD8+ T Cells via Trogocytosis

Regina Gary,* Simon Voelkl,* Ralf Palmisano,† Evelyn Ullrich,* Jacobus J. Bosch,* and Andreas Mackensen*

Upon specific interaction with APCs, T cells capture membrane fragments and surface molecules in a process termed trogocytosis. In this study, we demonstrate that human Ag-specific CD8+ T cells acquire the coinhibitory molecule programmed death ligand 1 (PD-L1) from mature dendritic cells (mDC) and tumor cells in an Ag-specific manner. Immature dendritic cells were less effective in transferring surface molecules onto CD8+ T cells than mDCs. Interestingly, trogocytosis of PD-L1 requires cell–cell contact and cannot be induced by uptake of soluble proteins obtained from mDC lysates. The transfer process is impaired by inhibition of vacuolar ATPases in T cells as well as by fixation of dendritic cells. Of importance, CD8+ T cells that acquired PD-L1 complexes were able to induce apoptosis of neighboring programmed death 1–expressing CD8+ T cells. In summary, our data demonstrate that human CD8+ T cells take up functionally active PD-L1 from APCs in an Ag-specific fashion, leading to fratricide of programmed death 1–expressing, neighboring T cells. The transfer of functionally active coinhibitory molecules from APCs onto human CD8+ T cells could have a regulatory role in immune responses. The Journal of Immunology, 2012, 188: 744–752.
may be involved in the immune escape of tumor cells. Multiple clinical studies have demonstrated that PD-L1 expression on tumors correlates with unfavorable prognosis (29, 30).

In this study, we investigated the Ag-specific transfer of PD-L1 from human APCs onto CD8⁺ T cells via trogocytosis and found that acquired PD-L1 strongly impaired T cell responses. PD-L1 acquisition may play a role in the limitation of Ag-specific T cell expansion and may also lead to T cell exhaustion in tumors and chronic infections.

Materials and Methods

**Cells and cytokines**

T cells were cultured in RPMI 1640 medium (PAN Biotec, Aidenbach, Germany) plus 10% human AB serum (PAN Biotec). The following recombiant human cytokines were used for differentiation of DCs: 500 U/ml GM-CSF (Bayer, Leverkusen, Germany), 25 U/ml IL-4 (Promokine, Heidelberg, Germany), 5 ng/ml TGF-β1 (Peprotech, Hamburg, Germany), 10 ng/ml IL-1β (Promokine), 1000 U/ml IL-6 (Promokine), 10 ng/ml TNF (Promokine), and 1 μg/ml PGE₂ (Enzo, Lörrach, Germany). Preparation of T cell growth factor was described previously (31).

**Cells and line cells**

Melan-A⁺ and gp100-specific CTL lines were generated, as described previously (32). Briefly, PBMCs from healthy donors were collected by leukapheresis, followed by gradient centrifugation (Pancoll; PAN Biotec). The study was approved by local ethics committee of the University of Erlangen. Informed consent was provided according to the Declaration of Helsinki. CD8⁺ T cells were enriched from PBMC using a negative isolation strategy (Miltenyi Biotec, Bergisch-Gladbach, Germany). Autologous DCs were generated from monocytes and cultured in complete medium plus 10% FCS, supplemented with GM-CSF, IL-4, and TGF-β. On day 6, GM-CSF, IL-4, TNF, IL-6, IL-1β, and PGE₂ were added. The culture was continued for additional 48 h, and mature DCs (mDCs) were pulsed with 30 μg/ml HLA-A2–binding Melan-A26–35 (ELAGIGILTV) or gp100209–217 (ITDQVPFSV) peptide (Bachem, Weil am Rhein, Germany) and 10 μg/ml human β₂-microglobulin (Scipac, Sitlingbourne, U.K.) for 2 h at 37°C in serum-free complete medium. Complete medium supplemented with 3% T cell growth factor was replenished twice per week. CD8⁺ T cells were restimulated weekly with Ag-pulsed DCs in 96-well U-bottom plates. T cell clones were isolated from primary CTL lines by single cell sorting (FACSAria; BD Biosciences, San Diego, CA) using appropriate MHC tetramers (TM). CD8⁺ T cell clones were expanded by repetitive stimulation with allogeneic irradiated EBV-transformed B cells, irradiated allogeneic PBMC, and PHA-L (1 μg/ml; Sigma-Aldrich, Munich, Germany).

The melanoma cell line Mel1300 (HLA-A²⁺, Melan-A⁺, gp100⁺) was cultured in complete medium with 10% FCS (PAA Laboratories, Pasching, Austria). The Mel1300 cell line was provided by M. Nishimura (Department of Surgery, Medical University of South Carolina, Charleston, SC). Expression of HLA-A2, Melan-A, and melanoma-associated chondroitin sulfate proteoglycan (MCSP) on Mel1300 cells was documented via flow cytometry analysis. For the trogocytosis assay, Mel1300 cells were incubated in the presence or absence of 200 ng/ml IFN-γ (Promokine). The lymphoblast cell line T2 (33) was obtained from American Type Culture Collection (Manassas, VA) and is characterized by an increased expression of 2-microglobulin. Next, target cells were stained with lipophilic membrane dye PKH67 (Sigma-Aldrich) for 20 min at room temperature (RT) and washed three times with complete medium plus 10% human AB serum. Harvested T cell clones and target cells were placed in 96-well U-bottom plates (Falcon; BD Biosciences) with an E:T ratio, unless otherwise noted, of 3:1 (7.5 × 10⁵ T cells plus 2.5 × 10⁵ target cells in 200 μl complete medium plus 10% human AB serum), incubated at 37°C, and harvested at indicated time. Harvested cocultures were washed with PBS (Life Technologies, Invitrogen, Darmstadt, Germany), resuspended before staining with antibodies and analyzed by flow cytometry.

For the preparation of DC lysate, mDCs were pulsed with peptides and labeled with membrane dye, as described before. Lysates were generated by repeated freeze-and-thaw cycles of a defined number of mDCs.

For blocking of trogocytosis, T cells or DCs were incubated with 0.1 μg/ml concanamycin A (CMA; Sigma-Aldrich) overnight, washed, and used for trogocytosis experiments. CMA revealed no toxic effect on human T cells and DC at the concentration used in our assays (data not shown).

For fixation of APCs, PKH67-labeled, Melan-A–pulsed mDCs were incubated for 30 min with 0.05% glutaraldehyde (Sigma-Aldrich) supplemented with 1% glucose, washed carefully, and used for trogocytosis experiments.

**MHC peptide TM, mab, and flow cytometry**

For conjugation, the HLA-A*0201 TM that had been folded around Melan-A26–35 or gp100209–217 were synthesized by Beckman Coulter (Fullerton, CA). For phenotypic analyses, the following mAbs were used: anti-C8-PerCP, anti–CD8-allophycocyanin, anti–CD8-allophycocyanin-Cy7, anti–CD3-PE-Cy7, anti–CD25-PE, and IgG1-PE (all from BD Pharmingen, Heidelberg, Germany); anti–PD-L1-PE, anti–PD-1-allophycocyanin, anti–PD-L1-biotin, unconjugated high-purified anti–PD-1 blocking mAb, and streptavidin-eFluor710 (all from eBioscience/NaturTec, Frankfurt, Germany); anti–CD209 (DC-SIGN)–allophycocyanin (R&D Systems, Wiesbaden-Nordenstadt, Germany); anti–MCSP-PE (Beckman Coulter); and anti–CD137–allophycocyanin and IgG2b-allophycocyanin (Caltag, Delberg, Germany), 5 ng/ml TGF-β (Scipac, Sitlingbourne, U.K.) for differentiation of DCs: 500 U/ml GM-CSF, 1000 U/ml IL-6 (Promokine), 10 ng/ml TNF (Promokine), and 1 μg/ml PGE₂ (Enzo, Lörrach, Germany).

**Trogocytosis experiments**

For trogocytosis experiments, APCs (DCs, T2, or Mel1300 cells) were pulsed for 2 h with 10 μg/ml relevant or irrelevant peptide and 10 μg/ml β₂-microglobulin. Next, target cells were stained with lipophilic membrane dye PKH67 (Sigma-Aldrich) for 20 min at room temperature (RT) and washed three times with complete medium plus 10% human AB serum. Harvested T cell clones and target cells were placed in 96-well U-bottom plates (Falcon; BD Biosciences) with an E:T ratio, unless otherwise noted, of 3:1 (7.5 × 10⁵ T cells plus 2.5 × 10⁵ target cells in 200 μl complete medium plus 10% human AB serum), incubated at 37°C, and harvested at indicated time. Harvested cocultures were washed with PBS (Life Technologies, Invitrogen, Darmstadt, Germany), resuspended before staining with antibodies and analyzed by flow cytometry.

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For fixation of APCs, PKH67-labeled, Melan-A–pulsed mDCs were incubated for 30 min with 0.05% glutaraldehyde (Sigma-Aldrich) supplemented with 1% glucose, washed carefully, and used for trogocytosis experiments.

**FIGURE 1.** Acquisition of membrane fragments, PD-L1, and CD209 from mDCs onto T cells by trogocytosis. A, Transfer of membrane fragments, PD-L1, and CD209 is Ag specific. Melan-A–specific CD8⁺ T cells were cocultured with PKH67-labeled mDCs pulsed with either Melan-A or irrelevant gp100 peptide. Acquisition of PKH67 membrane fragments, PD-L1, and CD209 was measured after 2 h of coculture with (upper panel) or without (lower panel) Ag recognition by flow cytometry (solid line). Dashed line in lower panel shows T cells before coculture as negative control. Histograms are gated on CD8⁺PKH67low lymphocytes. Shown is one representative of five independent experiments with similar results. B, Visualization of transferred membrane fragments and PD-L1 on T cells by confocal microscopy. mDCs were pulsed with specific Melan-A or irrelevant peptide, followed by PKH67 labeling (green). T cells were stained with PKH26 (red). After a coculture of 2 h with or without Ag recognition, cells were incubated with anti–PD-L1-biotin, followed by secondary streptavidin-eFluor staining (blue). Images were captured with a ×63 oil objective and 12-bit sequential scans. A ×5 zoom was used for high resolution single-cell images. Pictures are enhanced in brightness and contrast for improved printability.
Flow cytometry was performed on a FACSCanto II (BD Biosciences). T cells were gated on CD8*PKH67low cells to be distinguished from PKH67high-labeled APCs. Doubles were excluded via FSC-W gating. FACS data were analyzed with FlowJo software PC version 7.6 (Cелеza, Olten, Switzerland).

**Cell sorting and apoptosis assay**

Cell sorting of gp100-specific CD3*CD8* T cell clones for subsequent analysis after trogocytosis process was performed using a MoFlo cell sorter (Cytometry, Freiburg, Germany). For apoptosis experiments, cells were stained after coculture with Melan-A-TM for 30 min at RT, followed by staining with surface markers for 10 min at 4°C. Finally, cells were incubated with 5 μl annexin V FITC (Caltag) and 5 μl 7-aminoactinomycin D (7-AAD; BD Pharmingen) in 150 μl annexin-binding buffer (BD Pharmingen) at RT for 15 min in the dark, followed by immediate FACS analysis. For quantification of apoptotic T cells, the percentage of annexin V/7-AAD double-positive cells was calculated. Annexin V single-positive T cells cannot be counted as apoptotic cells, as annexin V is also binding to exposed phosphatidylserine on the surface of T cells upon T cell activation (34). For blocking of PD-L1–mediated apoptosis, PD-L1–acquiring cells cocultured with Melan-A–specific T cells were incubated with a highly purified anti-PDL1 mAb (10 μg/ml; eBioscience).

**Fluorescence microscopy**

Melan-A– or gp100-pulsed DCs were labeled with membrane dye PKH67, as described above, and T cells were labeled analogous with membrane dye PKH26 (Sigma-Aldrich). After coculture for 2 h at 37°C, cells were harvested, washed twice, and incubated with biotinylated anti-PD-L1 or corresponding IgG Ab for 15 min at 4°C. Cells were washed and incubated with streptavidin-eFluor710 nanocrystals for 20 min at 4°C. After four additional washing steps, cells were fixed for 15 min at RT with 3% paraformaldehyde (Sigma-Aldrich). Subsequently, cells were transferred on adherent SuperFrost Plus microscope slides (Langenbrück, Emmen- dingen, Germany). After settling and cell adherence, cells were washed with PBS. Almost dried slides were mounted with ProLong Gold Antifade Reagent (Invitrogen, Darmstadt, Germany). Slides were analyzed utilizing a laser-scanning microscope (Zeiss LSM 700, Jena, Germany) with the respective ZEN software (version 2009). Images were captured with a ×63 oil objective and in 12-bit mode. All three channels were corrected against bleed through and cross-talk before sequential scan for best separation was performed. Gain was adjusted accordingly and within the same magnitude for all channels. No signal enhancement was performed afterward, only linear unmixing and a deblurring filter with pixel range of 3. A 5× zoom according to Nyquist theorem was used for high resolution single-cell images.

**Transwell assay**

Ag-specific CD8* T cells were added to the bottom chambers of a 24-well tissue Transwell culture plate (Corning/Costar). Into the insert well, PKH67-labeled Ag-pulsed mDCs with or without Ag-specific PKH26-labeled T cells were placed to the top chamber as donor cells. Insert and bottom chambers were separated by a membrane with 5-μm pores, allowing soluble factors and membrane vesicles, but neither DCs nor T cells, to pass through.

**Statistical analysis**

All statistical analyses were performed with two-tailed, paired t test and are presented ±SD using GraphPad Prism version 5.02 (GraphPad, La Jolla, CA).

**Results**

CD8* T cells acquire PD-L1 and CD209 from APCs after Ag recognition via trogocytosis

First, we examined whether membrane-bound PD-L1 and CD209 on mDCs could be acquired by human CD8* T cells in an Ag-dependent manner via trogocytosis. CD209 has been described as a marker exclusively expressed by DC and absent from T cells, and therefore was chosen as control marker (35). Peptide-pulsed, PKH67-labeled mDCs were cocultured with a Melan-A–specific CD8* T cell clone. As shown in Fig. 1, T cells acquired high amounts of membrane patches, PD-L1, and CD209 after Ag recognition. In contrast, neither PD-L1, nor CD209, and just low amounts of membrane fragments were acquired when cocultured with control peptide-pulsed DCs. Biotinylation and PKH26 labeling of DCs confirmed the results obtained with PKH67 labeling, demonstrating a strong transfer of membrane fragments onto T cells after Ag-specific recognition and low Ag-independent acquisition of membrane molecules by T cells upon activation (data not shown). This Ag-independent transfer seems to be trig-
gered by preactivation, as nonstimulated T cells do not acquire membrane fragments (data not shown).

Next, we performed confocal microscopy to visualize the uptake of membrane patches and PD-L1 from APCs. PD-L1 expression was detected on mDCs, but not on T cells by secondary nanocrystal staining in the far red color spectrum (data not shown). After 2-h coculture of Melan-A–specific CTL with peptide-pulsed DCs, an Ag-specific uptake of PD-L1 and membrane fragments by CD8+ T cells was documented (Fig. 1B). Of importance, PD-L1 and membrane patches did not colocalize and could be detected on different regions of the T cell membrane. To verify that PD-L1 is not just upregulated on T cells after Ag-specific stimulation, Melan-A–pulsed, PKH67-labeled PD-L12 T2 cells were used as APCs for stimulation with Melan-A–specific T cells, demonstrating an uptake of membrane fragments and no upregulation of PD-L1 expression on T cells (Supplemental Fig. 1). Together, these data show that PD-L1 and CD209 are rapidly acquired after Ag-specific recognition of mDCs by CD8+ T cells.

Characterization of the Ag-specific transfer of PD-L1 and CD209 from APCs onto T cells

To assess the dynamics of the transfer of membrane fragments, PD-L1, and CD209, the kinetics of trogocytosis was documented at different time points. As shown in Fig. 2A, transfer of PD-L1, CD209, and membrane patches onto T cells is a process detectable already at 30 min after coculture. The acquisition of all three components peaked at 1.5–2 h and rapidly decreased after 3–4 h, reaching a plateau for ~20 h. Between 48 and 72 h of coculture, PD-L1, CD209, and membrane patches completely disappeared from the T cell surface. Of interest, the acquisition of membrane fragments, PD-L1, and CD209 revealed the same kinetics, suggesting a similar mechanism of capture. In contrast to this extrinsic PD-L1 signal upon trogocytosis, intrinsic PD-L1 expression peaked on day 4 after Ag-specific stimulation (data not shown).

To determine whether the amount of acquired molecules on T cells was higher when more APCs are targeted by specific T cells, T cells were cocultured with mDCs at different ratios and analyzed for trogocytosis of membrane fragments and PD-L1. As shown in Fig. 2B, an increase in membrane and PD-L1 uptake was observed up to a ratio of 1:1. Saturation was documented at a T cell:APC ratio of 1:3.

Role of APCs for Ag-specific trogocytosis

To investigate whether PD-L1 can also be acquired from other sources of APCs, Melan-A–pulsed immature DCs (iDCs) and

![FIGURE 3](https://example.com/figure3.png)
mDCs were compared in their capacity to induce trogocytosis. As shown in Fig. 3 A, expression of PD-L1 could be documented on iDCs, albeit to a lower extent. In contrast, CD209 was expressed on iDCs at similar or higher levels than on mDCs. Of interest, iDCs were only capable of transferring low amounts of CD209 and membrane fragments, but no PD-L1, onto T cells. In contrast, T cells cocultured with mDCs captured high amounts of all three components (Fig. 3B). Of note, Ag-unspecific uptake of membrane fragments from iDCs could not be detected (data not shown). These results demonstrate that iDCs are poor APCs in terms of triggering the transfer of surface molecules onto T cells in an Ag-specific manner.

Next, we asked whether tumor Ag-specific T cells are able to capture PD-L1, as well as membrane fragments from melanoma cells. To address this question, HLA-A2* Melan-A–expressing melanoma cells were analyzed for expression of PD-L1 and the Ag MCSP before and after IFN-γ treatment. MCSP was highly expressed independent on IFN-γ stimulation, whereas PD-L1 was only detectable after IFN-γ stimulation (Fig. 3C). After coculture of Melan-A–specific T cells with IFN-γ–treated melanoma cells, T cells acquired both PD-L1 and MCSP in parallel to membrane fragments, whereas no PD-L1 could be documented on T cells after coculture with untreated melanoma cells (Fig. 3D). These data demonstrate that PD-L1 is not exclusively transferred from mDCs, but also from tumor cells onto T cells upon Ag recognition.

Transfer of PD-L1 from APCs onto T cells requires cell–cell contact and is not mediated by soluble proteins from APCs

To assure that PD-L1 is transferred in a cell–cell contact-dependent manner, as defined for trogocytosis, in vitro Transwell experiments were performed, placing peptide-pulsed DCs in the presence or absence of PKH26-labeled Ag-specific T cells in the insert well separated from T cells in the bottom well. In both settings, transfer of PD-L1 and membrane fragments was absent (Fig. 4A).

Next, we asked whether T cells capture membrane fragments and molecules out of lysates after Ag-specific killing of APCs. When Melan-A–pulsed, PKH67-labeled mDCs were lysed via repeated freeze-and-thaw cycles, no transfer of PD-L1, but low amounts of membrane fragments were documented (Fig. 4B). These data confirm that Ag-specific trogocytosis requires cell–cell contact and is not induced via soluble proteins from APCs.

Ag-specific trogocytosis requires active interaction between APCs and T cells

Vacular ATPases (vATPases) were described to play an important role in membrane trafficking as well as endo- and exocytosis (36). We therefore asked whether CMA, a specific inhibitor of vATPases, is able to abrogate Ag-specific trogocytosis (37). To address this question, T cells or mDCs were exposed to CMA before coculture and T cells were analyzed for trogocytosis. Inhibition of vATPases in mDCs resulted in a nonsignificant, slightly reduced acquisition of membrane patches and PD-L1 by T cells. However, when T cells were pretreated with CMA, a strong inhibition of the transfer of PD-L1 (14.7 ± 5.1% mean fluorescence intensity [MFI] of maximal transfer compared with 4.4 ± 2.1% background MFI; p < 0.001) as well as the transfer of membrane fragments (23.9 ± 17.8% MFI of maximal transfer compared with 8.0 ± 7.3% background MFI; p < 0.01) was observed (Fig. 5A). These data demonstrate an important role of vATPases in the integration of surface molecules on T cells, yet attribute a minor role in the disruption of molecules out of DCs. Of interest, inhibition of trogocytosis in CMA-pretreated T cells had no impact on the upregulation of activation markers, such as CD25 and CD137 (Fig. 5B). Next, we asked whether active participation of mDCs is required in the transfer process. To address this question, Ag-

**FIGURE 4.** Mechanism of trogocytosis of PD-L1. A, Membrane fragments and PD-L1 are transferred in a cell–cell contact-dependent way. Melan-A peptide-pulsed, PKH67-labeled mDCs and PKH26-labeled, Melan-A–specific T cells were set into Transwell inserts separated by a membrane with 5-μm pores from bottom well. Melan-A–specific CD8+ T cells set in bottom well were analyzed after 2 h by flow cytometry (Transwell mDC + T cells). In the second setting, only Melan-A–pulsed, PKH67-labeled mDCs were set into insert well separated from Melan-A–specific CD8+ T cells in bottom well (Transwell mDC). Data were analyzed by flow cytometry. In bar graphs, MFI of Ag-specific maximum transfer was set to 100% (transfer). Inhibited transfer and no transfer are shown as percentage of maximum uptake of membrane fragments and PD-L1 of three independent experiments ± SD (** **p < 0.001, ***p < 0.01 paired t test). B, No impact of lysis of mDCs on acquisition of surface molecules. Melan-A–pulsed, PKH67-labeled mDCs were lysed by freeze-thaw cycles and incubated with Melan-A–specific CD8+ T cells for 2 h (lysat of mDC). As control, Melan-A–specific CD8+ T cells were cocultured with Melan-A peptide-pulsed, PKH67-labeled mDCs (vital mDC). In bar graphs, MFI of Ag-specific transfer was set to 100% as maximum. Inhibited transfer and no transfer are shown as percentage of maximum uptake of membrane fragments and PD-L1 of three independent experiments ± SD (ns p > 0.05, **p < 0.01 paired t test).
pulsed, PKH67-labeled APCs were fixed with glutaraldehyde and cocultured with Ag-specific T cells. As shown in Fig. 5C, fixation of mDCs resulted in a strong inhibition of the transfer of membrane fragments and PD-L1. Of importance, DC–T cell interaction was still functional because CD25 and CD137 were upregulated on T cells after coculture with glutaraldehyde-fixed mDCs. These data clearly indicate that acquisition is not merely induced by disruption of molecules out of the membrane, but depends on active interaction of APCs with T cells.

Acquired PD-L1 triggers apoptosis in neighboring T cells

To explore whether acquired PD-L1 on human CD8+ T cells is of functional relevance, gp100-specific T cells were stimulated with gp100 peptide-pulsed mDCs (PD-L1-acq+) or with anti-CD3/CD28 beads (PD-L12) according to the experimental setup depicted in Fig. 6A. Next, CD3+CD8+ cells were sorted out of both cultures and subsequently pulsed with Melan-A peptide to ensure interaction with Melan-A–specific neighboring T cells. Expression of acquired PD-L1 on gp100-specific T cells remained stable during the experimental procedure (Fig. 6B). Of note, PD-L1 was not upregulated on T cells after anti-CD3/CD28 mAb stimulation. Subsequently, sorted and Melan-A–pulsed gp100-specific T cells were cocultured with Melan-A–specific T cells, which were cocultured with PD-L1-acquiring gp100-specific T cells; revealed an increase in apoptotic cells of 28% compared with 12% in the control setting (coculture with PD-L12 gp100-specific T cells; \( p = 0.0251 \)). To confirm these findings, a blocking anti–PD-L1 mAb was added to the coculture, resulting in a significant reduction of the apoptosis to 15.3% (\( p = 0.0357 \)). Together, these data clearly emphasize that acquired PD-L1 on Ag-specific T cells is functional and can interact with its receptor PD-1 on neighboring T cells to induce apoptosis.

Discussion

Our study demonstrates a new role of trogocytosis in immune regulation via Ag-specific transfer of the functionally active coinhibitory molecule PD-L1 onto human CD8+ T cells. Compared
with previous reports on trogocytosis focusing mostly on the transfer of stimulatory molecules, we report here on acquisition of a relevant T cell-inhibitory molecule. HLA-G was described to render NK and T cells temporary to regulatory cells after acquisition of these nonclassical MHC class I molecules (15, 21). HLA-G is characterized by limited polymorphism and a restricted expression to immune-privileged sites such as fetal-maternal interface (38). In contrast, PD-L1 and its receptor PD-1 are expressed on a wide variety of human cells, indicating that acquisition of PD-L1 could have important regulatory function.

PD-L1, CD209, and membrane fragments are transferred after Ag recognition. The Ag-independent acquisition of low amounts of membrane fragments by T cells is exclusively found on activated T cells, suggesting trogocytosis could be tested as marker for T cell activation. Although human T cells are described to express PD-L1 upon activation, expression of mRNA and translation into protein take much longer than acquisition from APCs via trogocytosis. This phenomenon of trogocytosis has been described for a variety of molecules, such as costimulatory and coinhibitory molecules, lineage markers as CD4, and adhesion molecules (2). The reason for the transfer of these membrane fragments and molecules remains unclear. One reason for the transfer of PD-L1 may be recycling of the molecule, as discussed by Hudrisier and Bongrand (2). Neither PD-L1 nor CD209 is located at the immunological synapse; thus, it remains to be investigated which mechanisms regulate and trigger the transfer of molecules via trogocytosis.

Of note, trogocytosis was described as a phenomenon of very fast exchange of cell components compared with transfer via exosomes and nanotubes (39). This is in accordance with our kinetic studies of the uptake of membrane patches, PD-L1, and CD209. Kinetic studies indicate a reduction of transferred membrane molecules over time. The cause of this reduction could be internalization by the acceptor cells as it has been described for pMHC complexes and rituximab–Ab complexes (4, 40).

**FIGURE 6.** PD-L1 is still functional to trigger apoptosis after transfer. A, Experimental setup. For these experiments, T cells of a gp100-specific T cell clone were either cocultured with gp100-pulsed mDCs or stimulated with anti-CD3/CD28 beads for 2 h. Afterward, CD3+CD8+ T cells were sorted out of both cultures, the one population gp100(mDC)PD-L1acq+, and the other population gp100(beads)PD-L1−. These sorted T cells were pulsed with Melan-A peptide. Afterward, either gp100(mDC)PD-L1acq+ or gp100(beads)PD-L1− sorted T cells were cocultured with Melan-A–specific T cells from a CD8+ T cell culture (CTL) expressing PD-1. After 6 h, apoptosis rate was analyzed by annexin V and 7-AAD staining in CD3+CD8+ Melan-A T cells via flow cytometry.

B, PD-L1 maintenance of gp100 (mDC)PD-L1acq+ T cells during experimental procedures. Populations of both cocultures were monitored for PD-L1 after coculture, after cell sort, and after Ag pulsing. Histograms are gated on CD8+ lymphocytes. Shown is one representative of three independent experiments.

C, PD-L1 triggering apoptosis in neighboring T cells. After 6-h coculture of Melan-A–specific CTL with either gp100 PD-L1acq+ T cells or gp100 PD-L1− T cells, CD8+ Melan-A T cells were analyzed for 7-AAD and annexin V double-positive cells. Shown are dot plots from one representative of three independent experiments with similar results. Bar graph shows the x-fold increase of percental apoptosis rate compared with unstimulated CD8+ Melan-A T cells with data of three independent experiments ± SD (*p < 0.05, paired t test).
We also show that T cells express surface PD-L1 neither after coculture with Ag-pulsed PD-L1+ APCs, nor after stimulation with anti-CD3/CD28 beads, demonstrating that PD-L1 is not endogenously upregulated on T cells after Ag-specific activation with APCs in this time frame. The acquisition of molecules is restricted on the one side by the quantity of interactions with APC, and on the other side presumably by saturation of the integration of additional membrane fragments into the T cell’s surface. In summary, trogocytosis induces human CD8+ T cells with transient regulatory activity via acquired PD-L1 that triggers apoptosis in neighboring PD-1–expressing T cells.

Of interest, membrane fragments and surface molecules such as PD-L1 and CD209 are not equally acquired from immature and mature DCs, which might be explained by the higher expression of costimulatory molecules on mDCs than on iDCs (data not shown), mediating a stronger activation of T cells and therefore triggering trogocytosis. In accordance with this hypothesis, tumor cells, missing costimulatory molecules, transfer less surface molecules than mDCs. A very high expression of costimulatory molecules on mDCs suppresses the inhibitory effect of the PD-L1. In contrast, a shift causing imbalance with a predominant PD-L1 expression on mDCs results in an outweighing inhibitory signaling of PD-L1, as described in chronic infections (24). In case of PD-L1 acquisition by T cells, the inhibitory signaling of PD-L1 is dominant, leading to apoptosis of neighboring T cells.

Triggering apoptosis in neighboring PD-1–expressing T cells, trogocytosis of PD-L1 may represent a new immunoregulatory mechanism. PD-1 signaling is described to result in either anergy or T cell deletion, depending on the amount of Ag levels: low Ag levels cause deletion, and high Ag levels end up in anergy of T cells (41–43). Trogocytosis of pMHC complexes has been described in mice and humans (4, 5, 10); thus, T cells may present acquired pMHC complexes and PD-L1 to neighboring T cells in parallel. After clearance of Ag-presenting DCs and/or infected cells, the presence of just low amounts of Ag favors apoptosis mediated by PD-L1 and not anergy, which is triggered in the presence of high amounts of Ag. Moreover, transferred adhesion molecules such as CD209 onto T cells may temporarily support the interaction and cross-talk with neighboring T cells.

PD-L1 is acquired by T cells in a cell–cell contact-dependent manner and is not induced by an uptake of floating cell fragments after lysis of targeted DCs, suggesting that this is not a random process taking up surrounding molecules, but a directed transfer triggered by Ag recognition. Together, PD-L1 and CD209 transfer matches the characteristics of trogocytosis: the transfer is fast and dependent on cell–cell contact and on Ag recognition. Of note, bystander T cells are not triggered by neighboring T cells recognizing the APCs to acquire surface molecules (data not shown), demonstrating that trogocytosis is an Ag-specific process and thereby a selective tool to orchestrate immune responses. Moreover, we demonstrated that both inhibition of vATPases in T cells and fixation of APCs impair trogocytosis, suggesting that the trogocytosis process is controlled by membrane dynamics. This hypothesis is supported by Hudrisier et al. (44), who demonstrated that trogocytosis in T cells, but not in B cells, is dependent on an active actin cytoskeleton. vATPases are described to play a role in endocytosis, exocytosis, and membrane trafficking (reviewed in Ref. 36). Of interest, impairment of vATPases in mDCs has only a minor impact on the transfer of cell components from mDCs onto T cells, whereas blockade of vATPases in T cells results in a very strong inhibition of the trogocytosis process. These results indicate that vATPases play a role in the integration of disrupted molecules from neighboring cells into the plasma membrane. Although the transfer process is abolished by inhibition of vATPases, T cells still become activated. Of note, fixation of mDCs strongly inhibited the transfer process, supporting the idea of an active process between DCs and T cells and the prerequisite of a nonstatic surface of DCs for trogocytosis. Although inhibition of trogocytosis does not impair the upregulation of activation markers, resting T cells can be triggered to perform trogocytosis by prestimulation probably due to an increase in avidity of adhesion molecules upon activation (21).

The acquisition of PD-L1 by tumor-infiltrating T cells may spread the inhibitory function to the tumor environment. Many melanoma cell lines are described to upregulate PD-L1 upon IFN-γ exposition (45), hence in the case of an inflammatory immune response. As PD-L1 is described to play a role in chronic infections, acquired PD-L1 may contribute to exhaustion of T cell responses.

In conclusion, this study shows that coinhibitory PD-L1 can be transferred between immune cells that may influence the outcome of immune responses during infections and malignant diseases. Further understanding of the mechanism of trogocytosis and the physiologic relevance in immune regulation may have important implications for novel immune-based strategies.

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References
TRANSFER OF FUNCTIONAL PD-L1 ONTO HUMAN T CELLS


