

Trogocytic CD137 transfer causes an internalization of CD137 ligand on murine APCs leading to reduced T cell costimulation

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ABSTRACT

CD137 ligand (CD137L) is expressed on APCs and cross-links CD137, a powerful costimulatory molecule on T cells during cognate interactions, and thereby greatly enhances immune responses. We report that CD137 can be transferred from activated T cells and from tumor cells that express CD137 to other cells via trogocytosis. This trogocytic transfer is independent of CD137L expression by the recipient cell. However, if CD137L is present on the recipient cell, the transferred CD137 binds to CD137L and the CD137-CD137L complex becomes internalized. The removal of CD137L from the surface of APCs lowers their ability to costimulate T cells, as evidenced by a reduced IFN- γ secretion. Removal of CD137L on APCs by trogocytic transfer of CD137 occurs within 1 h and requires cell-cell contact and the continuous presence of CD137-expressing cells. Bidirectional signaling exists for the CD137 receptor/ligand system, because CD137L also signals into APCs. We propose that the trogocytic transfer of CD137 from activated T cells to APCs and the subsequent removal of CD137L from APCs is a physiologic regulatory mechanism that limits immune activity. Furthermore, we hypothesize that the trogocytic transfer of CD137 occurs in cancers and quenches the activity of APCs, contributing to the cancer cells escaping immune surveillance. Taken together, our findings demonstrate that the trogocytic transfer of CD137 leads to an internalization of CD137L on APCs and a reduction in immune activity. *J. Leukoc. Biol.* 97: 909–919; 2015.

Introduction

Immune homeostasis (i.e., the balancing of providing effective immune protection and avoiding autoimmunity), is essential for health. Accordingly, the immune system uses a range of mechanisms that regulate the activities of immune cells and immune responses. For example, the activity of costimulatory receptors such as CD28 or ICOS is kept in check by inhibitory receptors, such as CTLA-4 or PD-1 [1, 2]. In addition, cell surface receptors can be cleaved, and the resulting soluble

receptor isoforms can compete with the cell surface bound receptors for binding to the ligand [3]. Furthermore, soluble forms can also insert as dominant negative inhibitors into receptor multimers on the cell surface and disrupt signaling [4].

CD137 (4-1BB, TNFRSF9) is a member of the TNFR family and is expressed on activation by T cells, NK cells, and vascular endothelial cells [5–8]. The best characterized activity of CD137 is its T cell costimulatory activity, and CD137 agonists have been shown to enhance protective immune responses against viruses and tumors in mice [5–8].

CD137 ligand (CD137L), a member of the TNF family, is expressed by APCs, and APCs use the CD137 receptor/ligand system to costimulate T cell activity [9–12]. CD137L occurs as a soluble protein and as a transmembrane protein on the cell surface [13]. In the latter form, CD137L is able to transmit signals into the cells on which it is expressed, a process referred to as reverse signaling [14]. Bidirectional signaling (i.e., signaling through CD137 and CD137L) occurs when CD137-expressing and CD137L-expressing cells interact [15, 16]. Reverse signaling by CD137L into APCs enhances APC activity. It induces maturation of immature dendritic cells (DCs) [9–12] and even monocyte to DC differentiation [17–19].

The expression of CD137 or CD137L on most leukocytes, and even on some nonimmune cells, and the bidirectional signaling ability provide the basis for involvement of this receptor/ligand system in a wide range of immune activities, including hematopoiesis, T cell costimulation, APC activation, and B cell maturation [7]. Involvement in such diverse activities requires a complex regulatory network. Bidirectional signaling allows an immediate feedback signal and fine tuning of the activities of the interacting cells. Soluble forms are generated from CD137 by differential splicing, which antagonize the activities of cell surface CD137 [20–23]. Soluble forms of CD137L are enhanced in autoimmune disease and are prognostic factors

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for certain types of myeloid leukemia, implying a regulatory function [24, 25].

In addition to these mechanisms, we report the identification of a new regulatory mechanism. We show that CD137 can be transferred between cells by trogocytosis. If the recipient cell is an APC that constitutively expresses CD137L, the transferred CD137 will bind to CD137L, resulting in the internalization of the CD137-CD137L complex. The removal of CD137L from the cell surface diminishes the ability of the APCs to costimulate T cells. In addition, removal of CD137 from the T cell surface by trogocytosis diminishes their ability to become costimulated. These data identify a novel regulatory mechanism for the CD137/CD137L system and, potentially, also for other receptor/ligand systems.

MATERIALS AND METHODS

Mice

Female BALB/c, C57BL/6, and OT-1 mice aged 8–16 wk were used as a source of splenocytes, T cells, and B cells. The mice were specifically pathogen free and were maintained with free access to food and water in the animal care facility at the National University of Singapore in accordance with the institutional guidelines for usage of experimental animals.

Cell lines

The macrophage cell line RAW264.7, the B cell lymphoma line A20, and the melanoma cell line B16 were purchased from American Type Culture Collection (Manassas, VA, USA). The full-length murine CD137 cDNA with signal peptide was cloned into the pCDNA3.1/Zeo vector (Invitrogen, Life Technologies, Carlsbad, CA, USA). CD137-overexpressing A20 (A20-CD137) and -B16 (B16-CD137) cells were generated by stable transfection and selection using 0.1 $\mu\text{g}/\mu\text{l}$ zeocin (InvivoGen, San Diego, CA, USA) or 1 $\mu\text{g}/\text{ml}$ Geneticin (Life Technologies), respectively. Single cell cloning was performed twice in 96-well plates. The cells were used as a source of membrane-bound CD137. Control A20 (A20-vector) and B16 (B16-vector) cells were stably transfected with the empty vector pCDNA3.1/Zeo(+) or pCDNA3.1(+) (Invitrogen), respectively. Selection and cloning were performed the same as for the CD137-expressing A20 and B16 cells. RAW264.7 and A20 cells were cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS. B16 cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS. DC 2.4, a DC cell line generated from C57BL/6 mice, was provided by Dr. Kenneth L. Rock (University of Massachusetts Medical School, Boston, MA, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 2-mercaptoethanol (Sigma-Aldrich), 0.1 mM nonessential amino acid, and penicillin-streptomycin (Gibco, Life Technologies) at 37°C in 5% carbon dioxide. All cells were free of mycoplasma.

Preparation of splenocytes

The mice were euthanized by carbon dioxide inhalation. The spleens were aseptically removed from the abdominal cavity and minced through a 40 μm nylon cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) using a 5 ml syringe core in 10 ml PBS. RBCs were depleted using Tris-NH₄Cl lysis buffer (0.144 M NH₄Cl, 0.017 M Tris-HCl). Splenocytes were washed with PBS containing 2 mM EDTA and resuspended in RPMI 1640 medium.

Isolation of T cells from splenocytes

Splenic T cells were isolated by MACS using the mouse Pan T Cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, the fresh splenocytes were labeled with a cocktail of biotin-conjugated antibodies against CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119 expressed on non-T cells

(B cells, NK cells, DCs, macrophages, granulocytes, and erythroid cells), followed by anti-biotin microbeads. The cell suspension was passed through an LS column in a strong magnetic field, and the untouched T cells were collected in the effluent. The purity of the T cells was >90%, as determined by flow cytometry using a PE-conjugated anti-CD3 antibody (eBioscience, San Diego, CA, USA).

CD8⁺ T cells and B cells were positively selected by MACS using CD8 or CD19 microbeads (Miltenyi Biotec), respectively. In brief, freshly isolated splenocytes were labeled with CD8 or CD19 microbeads for 15 min at 4°C. The labeled cells were then loaded into the MACS column, which was placed in a strong magnetic field. After the negative fraction was eluted using MACS buffer, the column was removed from the magnetic field, and the positively labeled cells were flushed out of the column. T and B cells were cultured in RPMI (Sigma-Aldrich) supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mg/ml penicillin-streptomycin, and 50 μM 2-mercaptoethanol.

Activation of OT-1 T cells

DC 2.4 cells were cultured with A20-vector or A20-CD137 cells at a ratio of 1:2 for 18 h at 37°C. A20 cells were then depleted using EasySep Mouse CD19 Positive Selection Kit (StemCell Technologies, Vancouver, BC, Canada). Isolated DC 2.4 cells were fixed using 2% paraformaldehyde in PBS for 15 min at 4°C and were washed twice in PBS. The cells were resuspended in media at 10⁶ cells/ml and pulsed with SIINFEKL peptides at 1 $\mu\text{g}/\text{ml}$ for 4 h at 37°C. Pulsed DC 2.4 cells were cocultured with suboptimally activated OT-1 T cells, at a ratio of 1:4 or 1:40, in RPMI 1640 medium supplemented with penicillin-streptomycin and 10% FBS and seeded in 96-well round-bottom plates. The cultures were incubated at 37°C in 5% carbon dioxide, and the supernatants were harvested after 24 h. The supernatants were stored at -20°C before ELISA.

ELISA

IFN- γ was measured using the ELISA DuoSet (R&D Systems, Minneapolis, MN, USA). The tetramethylbenzidine substrate reagent pack (BD Biosciences) was used to visualize the positive reactions, which were evaluated at 450 nm. Each sample was measured in triplicate.

Antibodies and reagents

Biotin-conjugated, PE-conjugated, FITC-labeled, and unconjugated anti-mouse CD137 (clone 17B5), CD137L (clone TKS-1), CD3 (clone 17A2), CD11c (N418), CD19 (clone 1D3), CD45 (clone 30F11), F4/80 (clone BM8), CD40 (clone 1C10), CD86 (clone PO3.1) streptavidin-PE-Cy5, streptavidin-APC, and the respective isotype controls (rat IgG2a, rat IgG2b, and hamster IgG) were obtained from eBioscience. Biotinylated anti-CD137 pAb was purchased from R&D Systems. Anti-IgM+IgG+IgA antibody was purchased from Millipore (Billerica, MA, USA). Con A was obtained from Sigma-Aldrich. CFSE was obtained from Invitrogen. PHK26 was obtained from Sigma-Aldrich and 7-AAD cell viability staining solution from eBioscience. Monodansyl cadaverine (MDC) was obtained from Sigma-Aldrich.

Flow cytometry

The cells ($2-3 \times 10^5$) were stained with the respective fluorochrome-conjugated antibodies in PBS containing 0.5% FBS and 0.1% sodium azide (FACS buffer) for 1 h at 4°C in the dark. The cells were then washed twice and resuspended in 500 μl FACS buffer. If fixation was required, the cells were fixed with 1% paraformaldehyde for 1 h at 4°C. Flow cytometry was performed using either a FACSCalibur (BD Biosciences) with CellQuest data acquisition and analysis software, or a Cyan flow cytometer (Dako, Glostrup, Denmark) with Summit software, version 4.3. Nonspecific staining was controlled by isotype-matched antibodies. For CFSE-labeled cells, LSR Fortessa (BD Biosciences) was used to minimize spectral overlap.

Statistics

Quantitative data are presented as the mean \pm SD. Statistical significance was determined using 2-tailed unpaired Student's *t* test. *P* values < 0.05 were considered statistically significant.

RESULTS

CD137 is transferred to CD137L-expressing cells

To investigate the role of CD137 in tumor development, we transfected CD137 or the empty expression vector (pcDNA3.1/Zeo) into murine A20 B cell lymphoma cells and B16 melanoma cells and established stable cell lines (Fig 1A). Enforced expression of CD137 on A20-CD137 and B16-CD137 cells led to a decrease in constitutively expressed CD137L (Fig. 1A). The expression levels of CD137 and CD137L correlated inversely (Fig. 1B). For subsequent experiments, B16-CD137, clone 2 was used.

We considered and tested several potential explanations for the inverse relationship of CD137 and CD137L expression. One was that CD137 might bind to CD137L, making CD137L undetectable by flow cytometry. To determine the potential transfer of CD137 between cells, we used cocultures with the murine macrophage cell line RAW264.7, which expresses CD137L constitutively but does not express CD137 (Fig. 2A). RAW264.7 cells were distinguished from A20 by their expression of F4/80, a macrophage marker.

RAW264.7 cells were cocultured with the CD137-transfected B cell lymphoma cells (A20-CD137) or their CD137-negative counterpart (A20-vector). After an overnight incubation, the cells were stained for F4/80 and CD137 or CD137L. Of the RAW264.7 cells that were in contact with A20-CD137 cells, 10.8% stained positive for CD137 compared with only 1.8% of RAW264.7 cells that were cocultured with the A20-vector cells, indicating a transfer of CD137 from the A20-CD137 cells to the RAW264.7 cells (Fig. 2B). CD137L expression on RAW264.7 cells was greatly reduced, from 63.3 to 9.9%, after they were in contact with A20-CD137 cells (Fig. 2B).

The transfer of CD137 is not unique to A20 B cell lymphoma cells. After an overnight coculture of RAW264.7 cells with B16-CD137 cells, 94.8% of the RAW264.7 cells became CD137-positive. In contrast, only 6.7% of the RAW264.7 cells that were cocultured with B16-vector cells stained for CD137 (Fig. 2C). Again, the expression of CD137L on RAW264.7 cells decreased significantly from 94.9 to 36.7% after being in contact with the B16-CD137 cells (Fig. 2C).

CD137 transfer does not occur exclusively to RAW264.7 cells but also to other CD137L-expressing cells. When untransfected A20 cells, which express CD137L constitutively, were cocultured with B16-CD137 cells, the percentage of CD137-positive A20 cells increased from 1.3 to 66.5% (Fig. 2D). In parallel, CD137L staining by the recipient cells decreased from 35.9 to 20.1%. The same pattern could be observed with the MFI, which increased for CD137 and decreased for CD137L in all 3 cell coculture combinations (Fig. 2E).

That a transfer of CD137 to CD137L-expressing cells was observed in all 3 of these coculture systems indicates that it is a general phenomenon and might be a physiologic mechanism.

CD137 is transferred via trogocytosis

The transfer of membrane material, inclusive of cell surface proteins, between cells was described previously as trogocytosis [26–28]. Trogocytosis is a fast process in which membrane patches are acquired by 1 cell from another cell during cell-cell contact. Trogocytosis has been reported for T cells, DCs, APC,

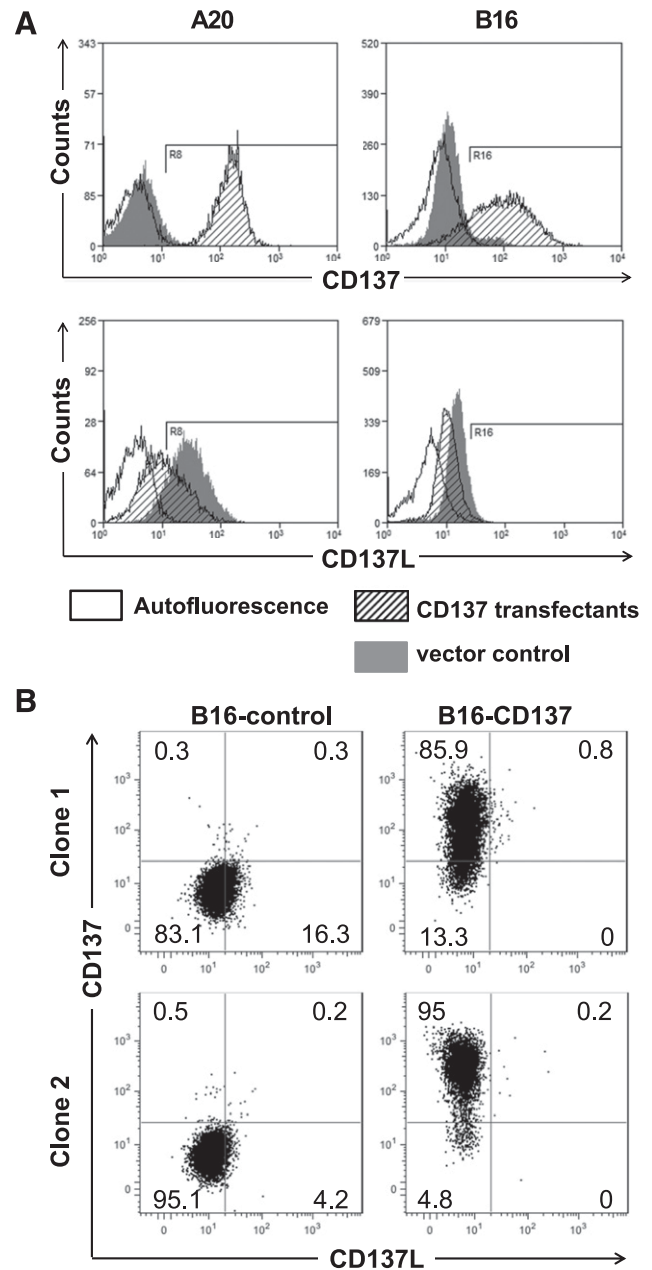


Figure 1. Reverse correlation of CD137 and CD137L expression in murine tumor cell lines. Murine A20 B cell lymphoma and B16 melanoma cells were transfected with CD137 or empty expression vector (pcDNA), and stable clones were established. Expression of CD137 and CD137L in these cells was evaluated by flow cytometry after staining with monoclonal antibodies against CD137 (clone 17B5) or CD137L (clone TKS-1). Expression of CD137 and CD137L in representative transfectants (A) and reverse correlation of CD137 and CD137L expression in multiple clones of B16-CD137 (B) are shown. Numbers indicate percentages of cells in quadrants.

NK cells, and tumor cells [27]. To determine whether only CD137 protein is transferred or is a part of a larger cell membrane transfer (i.e., by trogocytosis), we tested for the transfer of membrane material during coculture by labeling the cells with the fluorescent dyes PKH26 or CFSE before coculture (Fig. 3A). CFSE remains intracellular and is membrane-impermeable, and

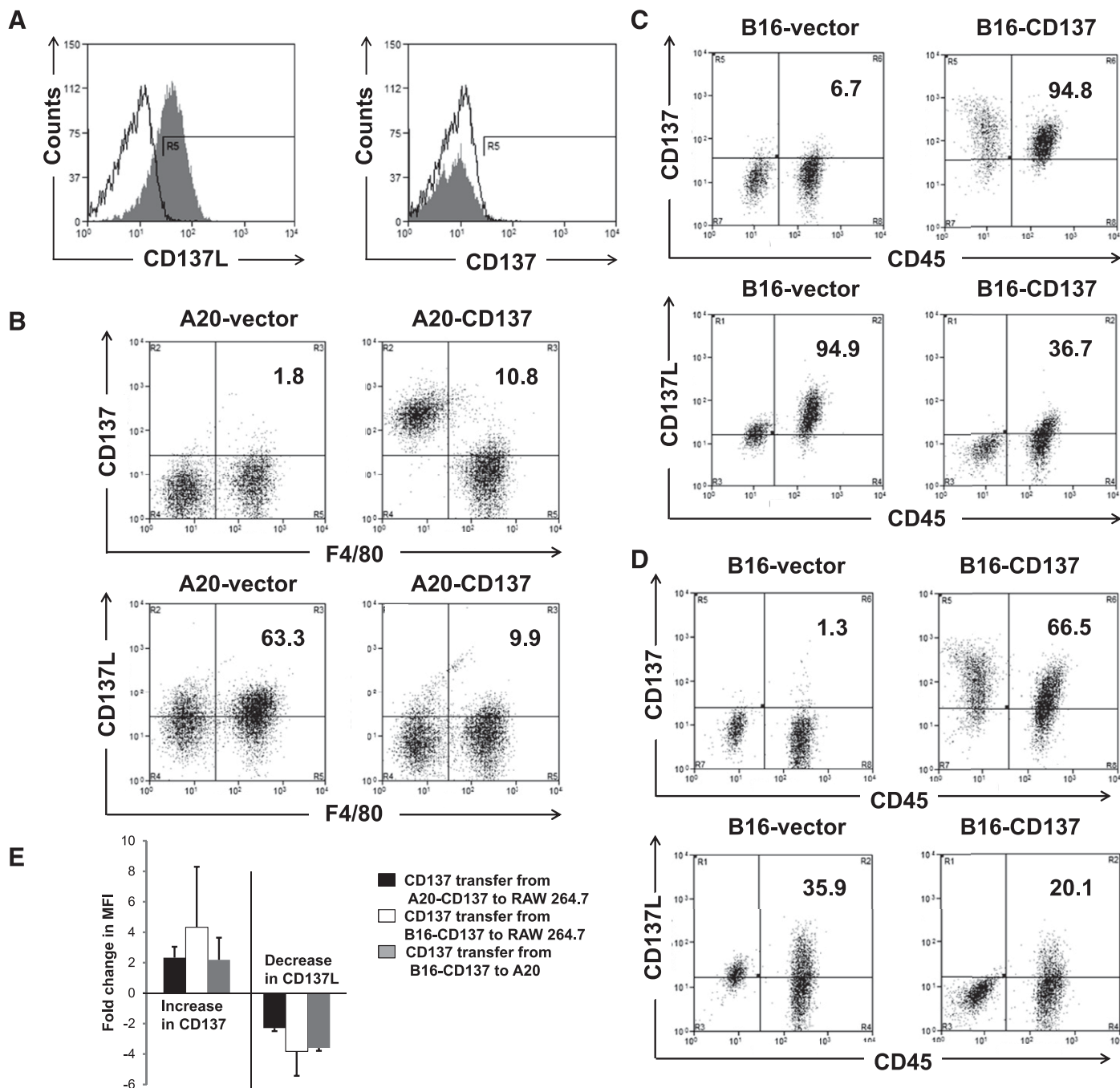


Figure 2. Transfer of CD137 to CD137L-expressing cells. (A) Expression of CD137 and CD137L on RAW264.7 cells detected by flow cytometry. (B–D) CD137 transfected cells (A20-CD137 or B16-CD137) or their CD137-negative counterparts (A20-vector or B16-vector) were cocultured with CD137L-expressing cells (RAW264.7 cells or untransfected A20 cells). (B) Coculture of A20-vector or A20-CD137 cells with RAW264.7 cells. Staining for the macrophage marker F4/80 was included to distinguish cocultured RAW264.7 cells from A20 cells. (C) Coculture of B16-vector or B16-CD137 cells with RAW264.7 cells. The hematopoietic cell marker CD45 was included to distinguish cocultured RAW264.7 cells from B16 cells. (D) Coculture of B16-vector or B16-CD137 cells with A20 cells. The hematopoietic cell marker CD45 was included to distinguish cocultured A20 cells from B16 cells. The numbers in the quadrants indicate the percentages of cells that became CD137⁺ as a result of coculture (upper panels) and cells that lost CD137L as a result of coculture (lower panels). (E) Changes in the MFI for CD137 and CD137L on the recipient cells of B–D. These experiments were conducted 3 times, with comparable results.

PKH26 can be transferred with membrane material from 1 cell to another during trogocytosis.

After an overnight incubation of PKH26-labeled A20-CD137 or A20-vector cells with CFSE-labeled RAW264.7 cells, most (>95%)

of the RAW264.7 cells had become PKH26-positive, indicating that they had acquired membrane fragments from the A20 cells (i.e., that trogocytosis had occurred) (Fig. 3B). RAW264.7 cells that were cocultured with A20-vector or A20-CD137 showed no

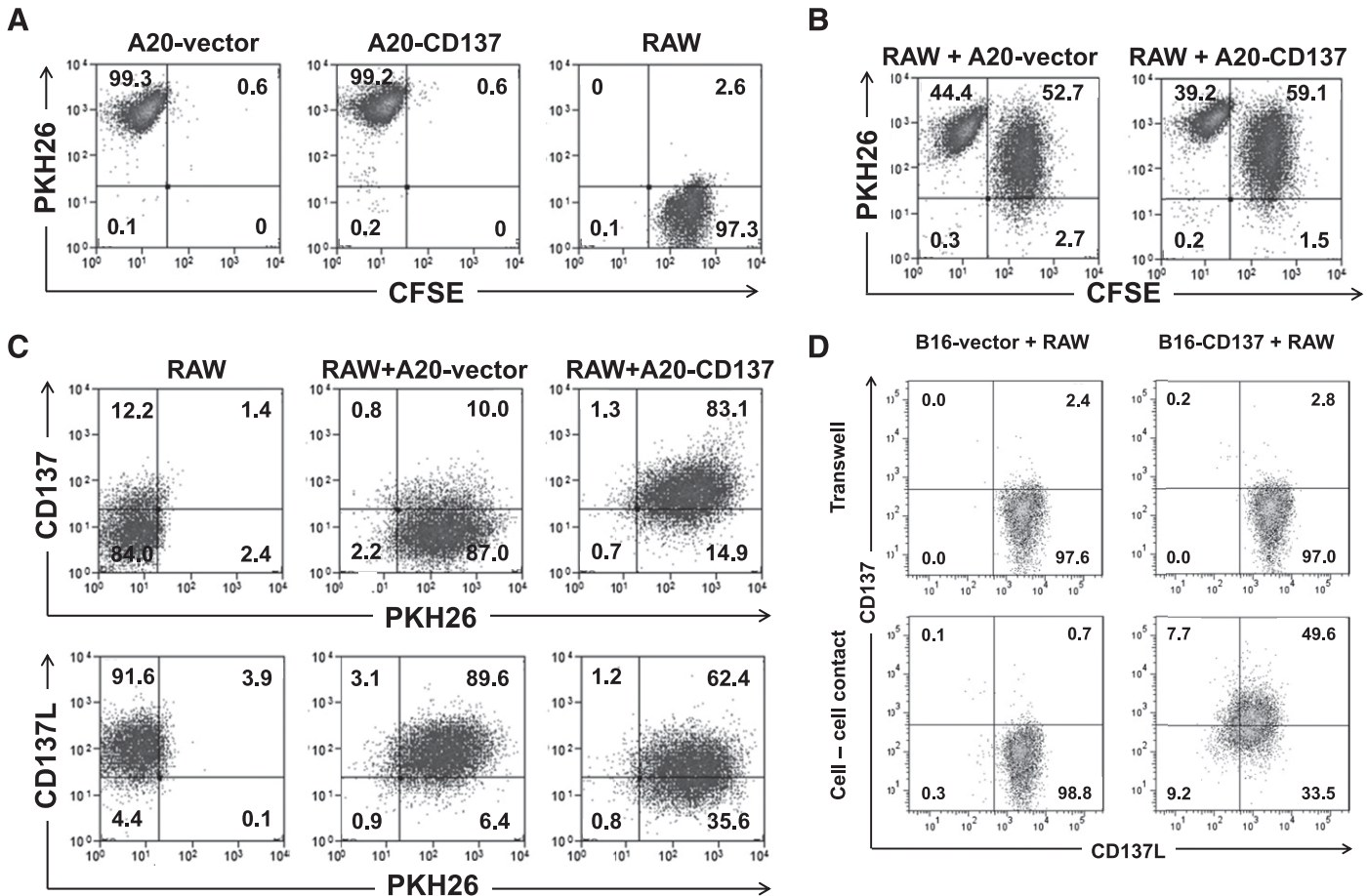


Figure 3. CD137 is transferred via trogocytosis. A20-CD137 or A20-vector cells were labeled with PKH26, and RAW264.7 cells were labeled with CFSE before cocultivation. The cells were evaluated by flow cytometry for the presence of the dyes before (A) and after (B) a 16 h coculture. (C) Cells of the coculture were stained with monoclonal antibodies for CD137 and its ligand, but only the CFSE-positive fractions (i.e., RAW264.7 cells) were gated and shown. (D) The CD137 transfer was evaluated in coculture experiments in which B16-CD137 cells and RAW264.7 cells were in contact (cell-cell contact) or were separated by a transwell system. Numbers indicate percentages of cells in quadrants. These experiments were conducted 2–3 times, with comparable results.

influence on the extent of trogocytosis, indicating that CD137 does not influence the extent of trogocytosis (Fig. 3B).

Significantly more RAW264.7 cells (83.1% vs. 10%) that were cocultured with A20-CD137 cells were positive for CD137, and significantly fewer (62.4% vs. 89.6%) were positive for CD137L, showing that the transfer of CD137 coincides with the transfer of membrane material during trogocytosis (Fig. 3C).

Because trogocytosis requires cell-cell contact, we asked whether cell-cell contact was also required for CD137 transfer. When control or CD137-expressing B16 cells were cocultured with RAW264.7 cells in a transwell chamber in which no contact between the cells was possible but in which sCD137 and other factors could pass between the chambers, no CD137 could be detected on the RAW264.7 cells (Fig. 3D). However when cell-cell contact was possible, transfer of CD137 from B16-CD137 cells to RAW264.7 cells occurred (Fig. 3D).

CD137L is internalized on transfer of CD137

Concomitant with the transfer of CD137 to CD137L-expressing cells, the latter lost CD137L (Fig. 2B–D). This was reminiscent of the findings from an earlier study on the CD28-B7 receptor-ligand

system. T cells were shown to take up B7 from APCs and then to internalize it [29]. Furthermore, internalization on binding of the ligand has also been shown for CD95 [30, 31] and TNFR1 [32]. Therefore, we tested whether CD137L also disappears by internalization.

RAW264.7 cells were cocultured with A20-CD137 or A20-vector cells. MDC, an endocytosis inhibitor, was added to inhibit internalization. After an overnight incubation, the cells were stained for F4/80 and CD137 or CD137L. CD137 had been transferred to RAW264.7 cells, as expected. When internalization was inhibited, significantly higher levels of CD137L (MFI 1705 vs. 848) and CD137 (MFI 1826 vs. 1548) could be detected on the surface of the RAW264.7 cells compared with the control cells, implying that CD137L is internalized on transfer of CD137 (Fig. 4A).

To rule out any involvement of phagocytosis in the CD137 transfer, we cocultured A20-vector and A20-CD137 cells with B16 cells, which lack phagocytic activity. On coculture with A20-CD137 cells, the B16 cells lost their CD137L expression and became CD137 positive (Fig. 4B). This demonstrates that phagocytosis is not required, and the transfer of CD137 can occur on nonphagocytic cells.

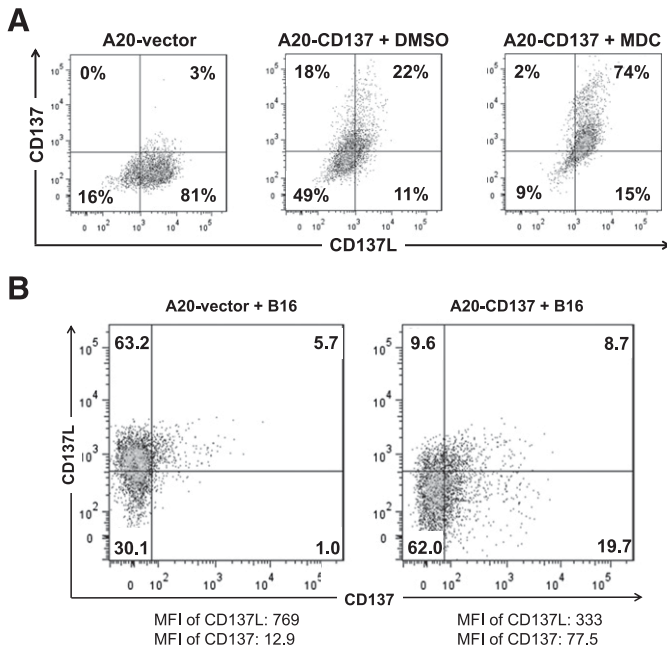


Figure 4. Endocytosis of CD137L. (A) CFSE-labeled RAW264.7 cells were cocultured for 16 h with A20-vector or A20-CD137 cells at a ratio of 1:1 in the presence of 50 μ M MDC or the solvent DMSO. The cells were stained for CD137 and CD137L and gated for CFSE (RAW264.7 cells). Numbers indicate percentages of cells in quadrants. (B) CFSE-labeled B16 cells were cocultured overnight with A20-vector or A20-CD137 cells at a ratio of 1:1 before being analyzed by flow cytometry. The numbers in the quadrants indicate the percentages of CD137⁺ and CD137L⁺ cells. This experiment was conducted twice, with comparable results.

CD137 transfer blocks CD137L-induced IFN- γ secretion

CD137 is a potent costimulatory molecule on T cells that becomes engaged during cognate interaction by CD137L-expressing APCs [5–8]. The transfer of CD137 to CD137L-expressing APCs could therefore lead to the disappearance of CD137L and impede the costimulatory activity of APCs. Hence, we tested the ability of the CD137L-expressing RAW264.7 cells in inducing IFN- γ secretion from T cells before and after coculture with CD137-expressing cells. When freshly isolated BALB/c splenocytes were suboptimally activated with anti-CD3 antibody and cocultured with CD137L-expressing RAW264.7 cells, they received costimulation, enhancing IFN- γ secretion by more than 10-fold (from 178 ± 94 to 2268 ± 184 pg/ml) (Fig. 5A). The addition of an antagonistic antibody against CD137L reduced the release of IFN- γ by more than one half (to 1053 ± 95 pg/ml), demonstrating the importance of the CD137-CD137L interaction for the induction of IFN- γ (Fig. 5A). RAW264.7 cells that had been in contact (pretreated) with A20-CD137 cells induced only one half as much IFN- γ release in splenocytes compared with control RAW264.7 cells that had been in contact with A20-vector cells (Fig. 5B). Identical results were obtained when splenocytes were substituted with purified T cells (Fig. 5C).

The inhibitory effect of CD137 transfer and the subsequent disappearance of CD137L was confirmed in an antigen-specific system using a DC line, DC 2.4. Expression of CD137L on DC 2.4 cells was greatly reduced on coculture with A20-CD137 cells,

but the expression levels of other costimulatory molecules such as CD86 remained unchanged (Fig. 5D). Subsequently, DC 2.4 cells were pulsed with OT-1 (SIINFEKL) peptide and used as APCs for T cell activation. T cells cocultured with DC 2.4 cells that were pretreated with A20-CD137 cells were less activated than the control T cells, as evidenced by the lower secretion of IFN- γ after 24 h (Fig. 5E). These data demonstrate that the transfer of CD137 to CD137L-expressing cells impairs their costimulatory activity.

Kinetics of CD137 transfer and CD137L internalization

To gain a better understanding of the influence of CD137 transfer on immune activities, we determined the time taken for CD137 transfer to occur and the duration of CD137L suppression.

When A20-vector or A20-CD137 cells were cocultured with RAW264.7 cells for different intervals, ranging from 10 min to 1 h, the reduction of CD137L on RAW264.7 cells could be observed as early as 10 min, with only 24% of the cells staining positive for CD137L compared with 54% before coculture (Fig. 6A). Because trogocytosis is a fast process, this result also supports the conclusion drawn in preceding sections (Fig. 3) that CD137 is transferred by trogocytosis.

To investigate how long the suppression of CD137L by transferred CD137 lasts, we cocubated A20-vector or A20-CD137 cells with RAW264.7 cells overnight and subsequently removed the A20 cells according to their CD19 expression using MACS. The purified RAW264.7 cells were then cultured for different time periods before being analyzed for CD137 and CD137L expression. RAW264.7 cells initially exposed to A20-CD137 cells showed an increase in CD137L expression after 3 h (17.3–46.2%), and, by 24 h, expression was completely restored (Fig. 6B). However, the continued presence of A20-CD137 cells sustained the suppression of CD137L expression on RAW264.7 throughout the 72 h of the experiment, albeit the suppression gradually weakened over time (Fig. 6B). To more precisely determine the time required for the reappearance of CD137L, we conducted a shorter time course experiment and found that 6 h after separation from A20-CD137 cells, the RAW264.7 cells exhibited normal CD137L levels. These data demonstrate that the CD137 transfer-induced disappearance of CD137L occurs quickly and lasts only a few hours and that long-term CD137L suppression requires the continuous presence of CD137-expressing cells (Fig. 6C).

CD137 transfer between primary lymphocytes

In the preceding experiments, the transfer of CD137 was investigated between cell lines and between cell lines and primary cells. To confirm that the transfer of CD137 also occurs between primary donor and recipient cells, T cells and B cells were isolated from murine splenocytes as sources for CD137 and CD137L, respectively. Because CD137 expression is strictly activation dependent, the T cells were activated with Con A, and 48 h after stimulation, the T cells expressed CD137 (Fig. 7A). CD137L is expressed constitutively by B cells, but its expression is enhanced after activation by anti-IgM+IgG+IgA antibody (Fig. 7A). B cells from CD137-deficient (CD137^{-/-}) mice were used as recipient cells to exclude endogenous expression of CD137. The

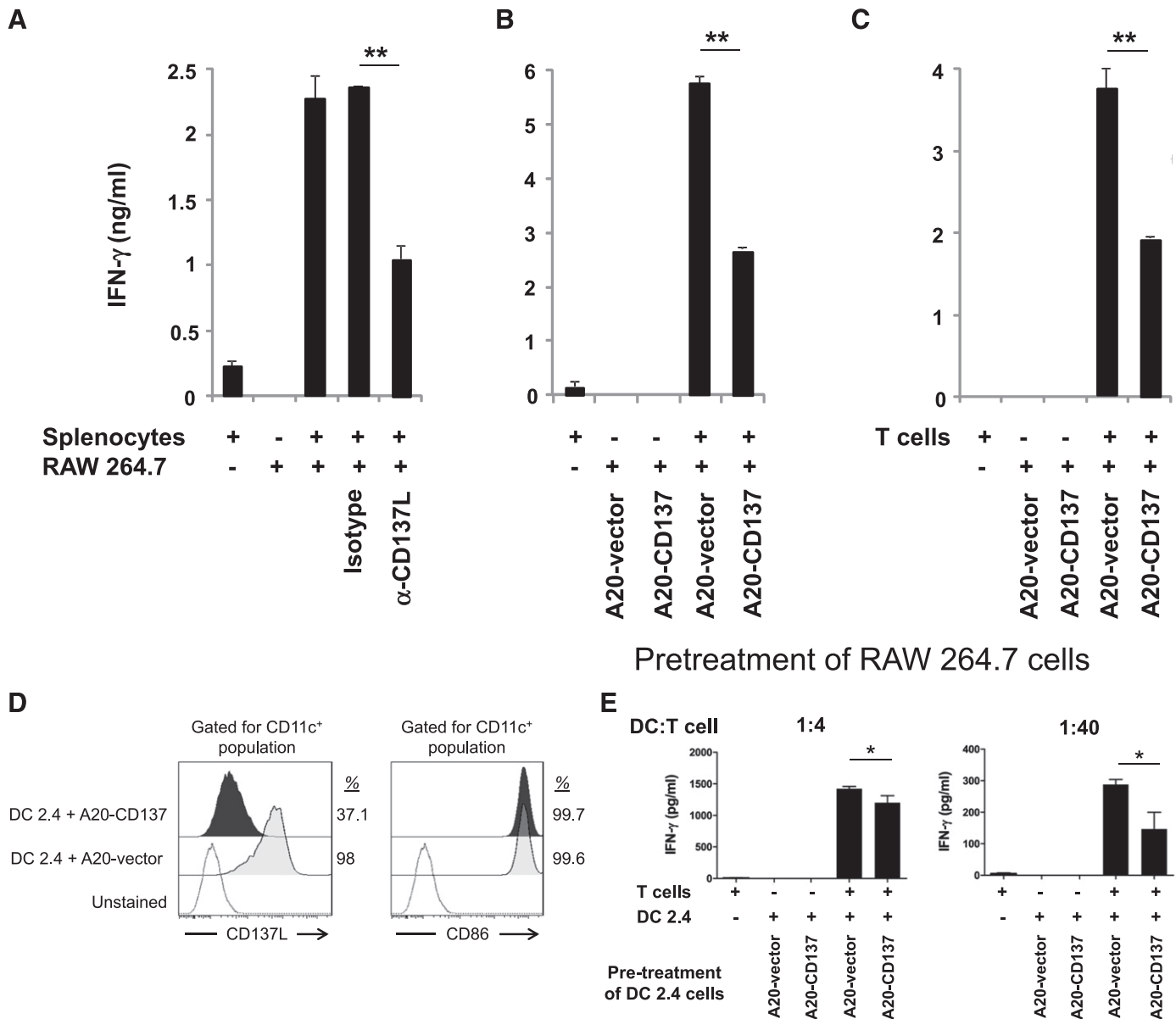


Figure 5. CD137 transfer blocks CD137L-mediated T cell costimulation. Mouse splenocytes were activated with suboptimal concentrations of anti-CD3 (1 μ g/ml, clone 17A2) and cocultured with RAW264.7 cells for 24 h. (A) A total of 5 μ g/ml antagonistic anti-CD137L antibody TKS-1 or an isotype control were added to the coculture. (B) RAW264.7 cells were cocultured with A20-vector or A20-CD137 cells at a ratio of 1:1 for 16 h and subsequently separated from the A20 cells before being used for coculture with the splenocytes at a ratio of 1:1 for 24 h. (C) The same conditions as in A, but purified T cells were used instead of splenocytes. Secretion of IFN- γ was measured by ELISA. Depicted are the mean \pm SD of triplicate measurements. This experiment was conducted 3 times, with comparable results. (D and E) DC 2.4 cells were cocultured with A20-vector or A20-CD137 cells at a ratio of 1:2 for 18 h and then separated from the A20 cells. (D) Surface expression of CD137L and CD86 was determined via flow cytometry. Data shown are from the gated CD11c⁺ population. (E) Pretreated DC 2.4 cells were pulsed with SIINFEKL peptide at 1 μ g/ml for 4 h at 37°C and cocultured with suboptimally activated OT-1 T cells at a ratio of 1:4 or 1:40 for 24 h. Secretion of IFN- γ was measured by ELISA. Depicted are the mean \pm SD of triplicate measurements. This experiment was conducted 2 times, with comparable results.

B cells were then cocultured overnight with T cells from either WT or CD137^{-/-} mice and thereafter stained for CD137 and CD137L. B cells cocultured with WT T cells showed a significant reduction of CD137L staining compared with B cells that were cocultured with CD137^{-/-} T cells (5.8% vs. 33.6%) (Fig. 7B). In contrast, 23.9% of the B cells became CD137-positive after encountering WT T cells, but B cells cocultured with CD137^{-/-} T cells remained negative for CD137. These data show that

endogenously expressed CD137 can be transferred to cells expressing endogenous CD137L.

DISCUSSION

The results of the present study have identified the trogocytic transfer of CD137 and its immunoregulatory effects. We found the transfer of CD137 to be dependent on cell-cell contact, to

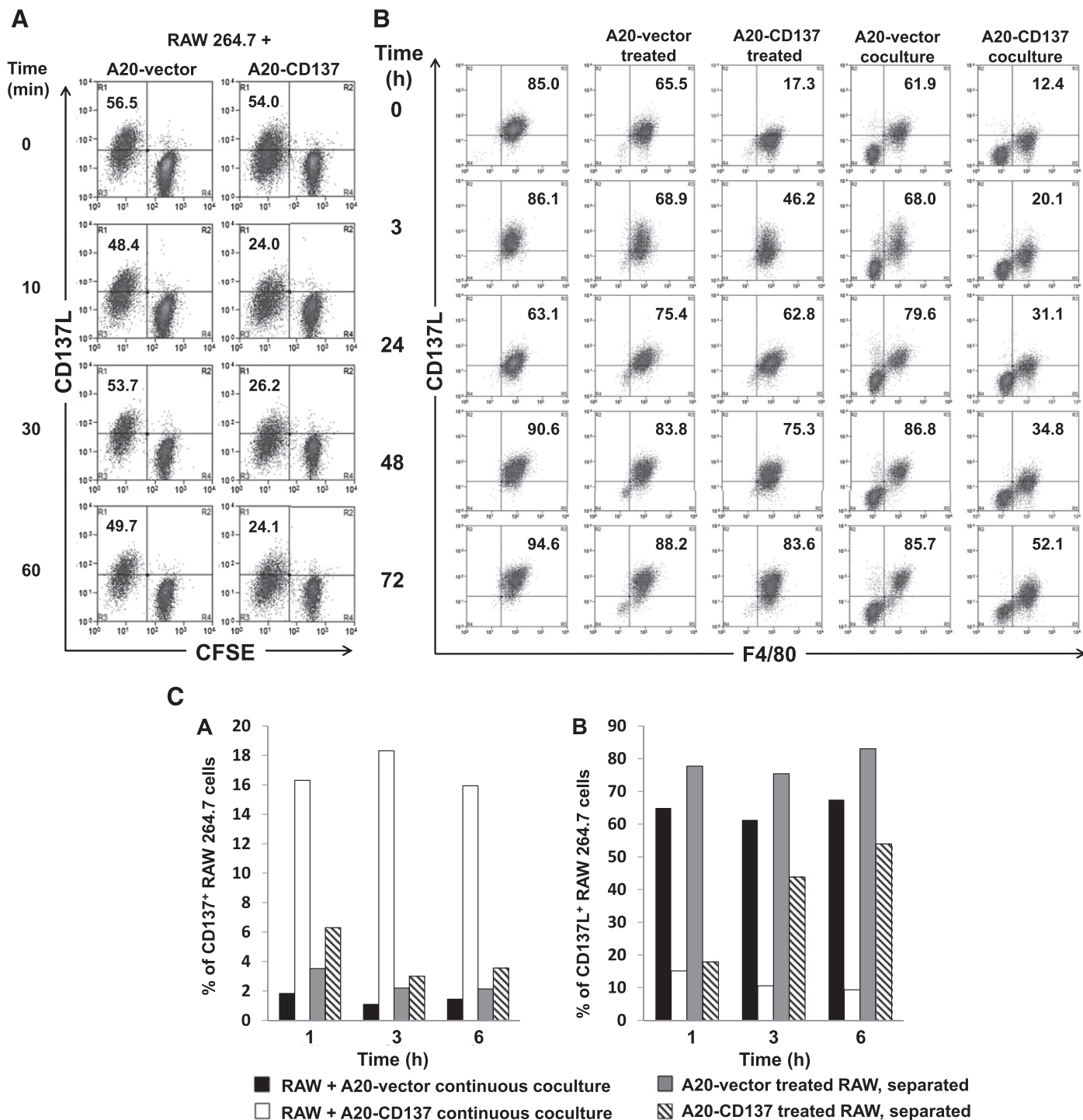


Figure 6. Kinetics of the CD137 transfer. (A) RAW264.7 cells were cocultured with CFSE-labeled A20-vector or A20-CD137 cells for intervals ranging from 10 min to 1 h. (B) RAW264.7 cells were cocultured (treated) with A20-vector or A20-CD137 cells overnight and separated from A20 cells by negative selection for CD19 using MACS. Isolated RAW264.7 cells were then cultured at different intervals, and expression of CD137 and CD137L was analyzed by flow cytometry. Untreated RAW264.7 (first column from left) and RAW264.7 cells in the continuous presence of A20-vector or A20-CD137 cells (last 2 columns from right, labeled “coculture”) were included as controls. (C) Same experimental conditions as in B, but the frequencies of CD137 (left) or CD137L (right) expressing RAW264.7 cells in the different treatment groups are shown for 1, 3 and 6 h after A20 and RAW264.7 cell separation. Expression of CD137 and CD137L on RAW264.7 cells was evaluated by flow cytometry. These experiments were conducted 2 times, with comparable results.

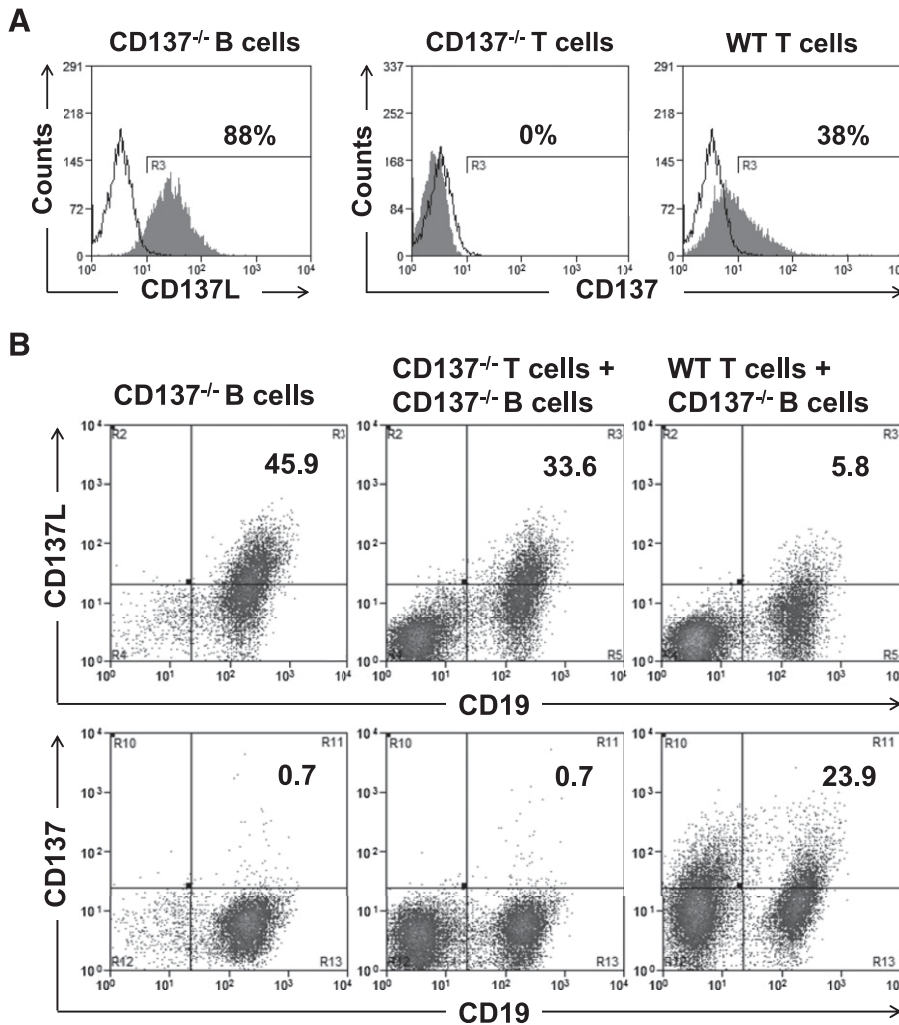


Figure 7. CD137 is transferred between primary T and B cells. Primary B cells were isolated from CD137^{-/-} mice and activated with 10 μ g/ml anti-IgM+IgG+IgA and 1 μ g/ml anti-CD40. CD8⁺ T cells were isolated from WT or CD137^{-/-} mice and activated with 2 μ g/ml Con A. (A) At 2 d after stimulation, expression of CD137L and CD137 was evaluated on B and T cells, respectively. Open histograms, isotype control; gray histograms, CD137L or CD137 staining, as indicated. (B) Primary CD137L-expressing B cells were cocultured overnight with primary WT or CD137^{-/-} CD8⁺ T cells, and expression of CD137 and CD137L was quantified by flow cytometry. Anti-CD19 was used to discriminate B cells from T cells, and 7-AAD exclusion was applied to gate for live cells. This experiment was conducted 3 times, with comparable results.

occur within 1 h, and the transferred CD137 to be of limited permanence on the recipient cell. Furthermore, the transfer of CD137 coincides with a general membrane transfer and is unspecific, because it occurs in the absence of CD137L on the recipient cell and is not reduced by the presence of CD137L-blocking antibodies. These are all characteristics of trogocytosis [33].

Trogocytosis is an extensive phenomenon that occurs both in vitro and in vivo, and its immune modulating effects have been well documented [34]. The uptake of CD86 by inducible regulatory T cells from DC was shown to augment their immune suppressive capacity [35]. Similarly, MHC class II molecules could be transferred from DC to NK cells, thereby negatively regulating CD4⁺ T cell responses [36]. Such reports clearly indicate a functional role for trogocytosis in immune regulation.

The trogocytic transfer of CD137 also occurs between human cells, where it can reduce immune activation [37]. CD137 is ectopically expressed by Hodgkin Reed-Sternberg cells, the malignant cells that cause Hodgkin's lymphoma [38, 39]. Hodgkin Reed-Sternberg cells derive from B cells that express CD137L constitutively and therefore costimulate CD137-expressing tumor infiltrating T cells. Hodgkin Reed-Sternberg cells gain a selection advantage if they reduce expression of

CD137L. We have shown that ectopically expressed CD137 binds to CD137L and that the resulting complex becomes internalized thereby reducing immune activation in the tumor microenvironment. Hodgkin Reed-Sternberg cells also transfer CD137 to surrounding APCs, which leads to the disappearance of CD137L also from APCs, thereby further contributing to the escape of Hodgkin's lymphoma from immune surveillance [38]. It is unlikely that Hodgkin's lymphoma established trogocytic CD137 transfer as a novel tool for immune escape. Rather, it might have used an existing physiologic mechanism of immune regulation. One limitation of our study was the use of cell lines, because cell lines can show artifacts that will not be observed in vivo. Despite that, the existence of such trogocytic CD137 transfer can be supported by the reproducibility of our results in repeat experiments ($n = 2-3$) and the identical results with different donor and recipient cell lines. Furthermore, we have also established the occurrence of trogocytosis of CD137 between primary T and B cells.

The physiologic effect of the CD137 transfer is an inhibition of immune responses by several mechanisms. First, the cells with the highest expression of CD137 are activated T cells, NK cells, and vascular endothelial cells [7, 40]. CD137 crosslinking enhances the activation of these cells. Accordingly, a depletion of CD137 by

a transfer to other cells would reduce their responsiveness to stimulation by CD137L-expressing APCs.

Second, if the recipient cell does express CD137L, such as is the case for APCs, the transferred CD137 will bind to the endogenous CD137L and the CD137-CD137L complex will be internalized. Because the bidirectional signal transduction exists in the CD137 receptor/ligand system, an internalization of CD137L by trogocytic transfer of CD137 would also make the APCs insensitive to activation or costimulation through CD137L.

Third, the trogocytic transfer of CD137 also limits the activity of third party cells that are not involved in the trogocytic transfer as donor and recipient cells. Because the CD137-CD137L complex becomes internalized, both molecules are no longer available to interact with their partner molecule on other cells and to initiate activating signals. For example, the transfer of CD137 from activated T cells to DCs during cognate interaction can reduce the ability of these DCs to stimulate NK cells or T cells in subsequent interactions.

The transfer of CD137 and the subsequent binding to CD137L occurs within 1 h and is therefore within the time frame of cognate T cell-DC interactions [41]. Equally fast is the complex formation between CD137 and CD137L and the disappearance of the CD137-CD137L complex. However, the reemergence of CD137L on the APCs also occurs within a few hours after the separation of the APCs from the CD137-expressing cells. Therefore, this regulatory mechanism would be initiated right after an activated T cell engages with an APC, persists while the cognate interaction lasts, and ends soon after the 2 cells disengage. A similar kinetic has been found for the absorption of molecules from APCs by T cells and their internalization, which also occurs within 1 h [29].

It is an open question whether CD137 also becomes internalized after a transfer to cells that do not express CD137L or whether it will remain for a longer time on the surface of CD137L-negative recipient cells. In the latter context, it might be possible that these newly CD137⁺ cells acquire the ability to stimulate APC via reverse signaling through CD137L. Furthermore, it might be that the transferred CD137 signals and changes the activities of the recipient cells. A precedent case has been reported for inhibitory Ig-like transcript 2 receptor. After transfer by trogocytosis, the receptor integrates into the membrane, signals, and elicits functional responses in the recipient cells [42].

The CD137 receptor/ligand system is subject to a complex network of regulation. The expression of CD137 is activation dependent [43]. In addition, bidirectional signaling is present, such that both the receptor and the membrane-bound ligand transmit signals into the respective cells on which they are expressed [16]. Furthermore, CD137 and CD137L both have soluble forms. Soluble CD137 antagonizes the activity of cell surface-expressed CD137; the activity of soluble CD137L is not known [21, 23]. Our work adds the transfer of CD137 by trogocytosis and the internalization of CD137-CD137L complexes as an additional mechanism. It remains to be determined what significance the regulatory mechanism of trogocytic CD137 transfer has compared with the other regulatory mechanisms of the CD137 receptor/ligand system.

We propose that the trogocytic transfer of CD137 from activated T cells to APCs and the subsequent removal of CD137L

from APCs is a physiologic regulatory mechanism to limit immune activity. Because many parallels exist between TNF and TNFR family members, it should be of interest to investigate whether the transfer of a receptor to a ligand-expressing cell and the subsequent internalization of the receptor-ligand complex also occur for other members of the TNF and TNFR families.

AUTHORSHIP

Z.S. designed and performed experiments, analyzed data and wrote the manuscript. Z.H., W.L.P., E.N., and L.K.K. designed and performed experiments and analyzed data. H.S. designed the project and wrote the manuscript.

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